Regulated Function of Oxygen Sensing HIF Prolyl-4-Hydroxylases

---

Dissertation

zur

Erlangung der naturwissenschaftlichen Doktorwürde (Dr. sc. nat.)

vorgelegt der

Mathematisch-naturwissenschaftlichen Fakultät

der

Universität Zürich

von

Katarzyna Józefa Nytko

aus Polen

Promotionskomitee

Prof. Dr. Roland H. Wenger (Vorsitz)
Dr. Daniel P. Stiehl (Leitung der Dissertation)
PD Dr. Gieri Camenisch
Prof. Dr. Christian Grimm
Dr. Felix Oehme

Zürich, 2011

This work has been performed under the supervision of:

Dr. Daniel P. Stiehl
and
Prof. Dr. Roland H. Wenger

at the Institute of Physiology/Zürich Center for Integrative Human Physiology (ZIHP), University of Zürich, CH-8057 Zürich, Switzerland

To my brother

1. Abbreviations:

ASC	ascorbate
AKAP12	A-kinase anchor protein 12
ALAS2	5-aminolevulinate synthase 2
ARNT	aryl hydrocarbon receptor nuclear translocator
ATF-4	activating transcription factor 4
BNIP3	BCL2/adenovirus E1B interacting protein 1
CAIX	carbonic anhydrase IX
CAD	carboxyterminal transactivation domain
CBP	CREB binding protein
CDR2	onconeuronal cerebellar degeneration-related protein 2
CHO	chinese hamster ovary
CPX	ciclopirox olamine
DHA	dehydroascorbate
DMOG	dimethyloxalylglycine
DTT	dithiothreitol
DFX	desferrioxamine
EDTA	ethylenediaminetetraacetic acid
EGL-9	Egg-laying abnormal-9
EPAS	endothelial PAS protein
EPO	erythropoietin
EDNRB	endothelin receptor type B
FCS	fetal calf serum
FH	fumarate hydratase
FIH	factor inhibiting HIF
FKBP38	FK506-binding protein 38
GLUT1	glucose transporter 1
GLRX	glutaredoxin
GSH	L-γ-glutamyl-Lcysteinyl-glycine, glutathione
GST	glutathione-S-transferase
GULO	gulono-1,4-lactone oxidase
HAT	hypoxantine, aminopterin, thymidine

HIF	hypoxia-inducible factor
HLH	helix-loop-helix
HSP90	heat shock protein 90
IC50	inhibitory concentration 50%
ID	inhibitory domain
IGF-2	insulin growth factor 2
IOP1	iron-only hydrogenase-like protein 1
IKKβ	inhibitor of NF-Kb kinase-β
IL-1β	interleukin 1β
ING4	inhibitor of growth family member 4
IPAS	inhibitory PAS protein
IPC	ischemic preconditioning
IRP2	iron regulatory protein 2
KIF1Bβ	kinesin family member 1Bβ
L28	ribosomal protein L28
OS-9	osteosarcoma amplified 9
PAS	Per/Arnt/Sim
PHD	prolyl-4-hydroxylase domain protein
MAGE-11	melanoma antigen 11
MAPK	mitogen-activated protein kinase
MMP3	matrix metallopeptidase 3
MnTMPyP	manganese(III) tetrakis(1-methyl-4-pyridyl) porphyrin pentachloride
MORG1	mitogen-activated protein kinase organizer 1
mRNA	messenger ribonucleic acid
NTA	nitrilotriacetic acid
NAC	N-acetyl cysteine
NAD	aminoterminal transactivation domain
NF-κB	nuclear factor 'kappa-light-chain-enhancer' of activated B-cells
NO	nitric oxide
N-OG	N-oxalylglycine
NOS	nitric oxide synthase

NDRG1	N-myc downstream regulated gene 1
ODDD	oxygen dependent degradation domain
PDGF	platelet derived growth factor
PDK1	pyruvate dehydrogenase kinase, isoenzyme 1
REDD1	regulated in development and DNA damage response 1 protein
ROS	reactive oxygen species
RLU	relative luciferase units
RpB1	ribonucleic acid polymerase II subunit B1
S12	ribosomal protein S12
SDH	succinate dehydrogenase
SEM	standard error of the mean
Sf9	Spodoptera Frugiperda 9
SIAH2	seven in absentia homolog 2
SOD	superoxide dismutase
SVCT1	sodium-dependent vitamin C transporter 1
SVCT2	sodium-dependent vitamin C transporter 2
TGF-β1	transforming growth factor-β 1
TMB	3,3',5,5'-tetramethylbenzidine
TRiC	tailless complex polypeptide -1 ring complex
TNFα	tissue necrosis factor
VBC	von Hippel Lindau, Elongin B, Elongin C
VEGF	vascular endothelial growth factor

2. Zusammenfassung

Eine genaue Rezeption des zellulären Sauerstoffgehalts durch Prolyl-4-Hydroxylase Domänen (PHD) enthaltende Sauerstoff-Sensorproteine ist für viele physiologische Prozesse im menschlichen Körper von grosser Bedeutung. Dieselben Signalwege spielen aber auch bei der Ausbildung von Krankheiten eine entscheidende Rolle. Hypoxie-induzierbare Faktoren (HIFs) sind das vorrangige Hydroxylierungssubstrat für PHDs und fungieren als Sauerstoff-regulierte Transkriptionsfaktoren, indem sie die Expression von mehr als 100 Zielgenen kontrollieren. Die wichtigsten Beispiele für HIF-regulierte Genprodukte sind unter anderem das für die Neubildung von Erythrozyten verantwortliche Erythropoietin (EPO), aber auch pro-angiogene Wachstumsfaktoren wie VEGF und Proteine des zellulären Glukose-Stoffwechsels (z.B. GLUT1, PDK1). Die enzymatische Aktivität von PHDs ist unmittelbar an die Verfügbarkeit von Sauerstoff gekoppelt, aber auch andere Kofaktoren und Kosubstrate beeinflussen ihre spezifische Hydroxylierungskapazität. Die Nutzung eines Hydroxylierungsassays als rekonstituiertes System erlaubte uns, die Abhängigkeit aller drei PHD Isoformen von verschiedenen einfachen, potentiell inhibierenden oder aktivierenden Substanzen („small molecules“) zu untersuchen. Während die enzymatische Aktivität von PHDs in Gegenwart von Eisen(II), 2-Oxoglutarat und Ascorbat dosisabhängig gesteigert war, inhibierten Substanzen wie Succinat oder polyphenolische Antioxidantien die Hydroxylierungs-aktivität der PHDs. Übergangsmetalle wie Kobalt(II) und Zink(II), chelierende Agenzien (DFX, EDTA) und reaktive Sauerstoffspezies (Wasserstoffperoxid) waren weitere effiziente Hemmstoffe der PHDs. Mechanistisch funktionieren diese Substanzen zumindest teilweise durch die Abreicherung oder Inaktivierung eines oder mehrerer der für die Hydroxylierungs-reaktion notwendigen Kofaktoren. In diesem Zusammenhang konnten wir zeigen, dass Kupfer(II), jedoch nicht Kobalt(II) oder Eisen(II), die Oxidation von Ascorbat zu Dehydroascorbat katalysieren kann, wobei letzteres keinen aktivierenden Einfluss mehr auf die PHDs ausübt. Eine Mangelversorgung mit Ascorbat (Vitamin C) verursacht die als Skorbut bekannte Systemerkrankung mit unzureichender Quervernetzung des Kollagens im Bindegewebe, der eine verminderte Aktivität der Kollagen-Prolyl-4-Hydroxylasen zugrunde liegt. Da PHDs zur gleichen Untergruppe von Enzymen wie Kollagen-Prolyl-4-Hydroxylasen gehören, wurde in

dieser Arbeit ein möglicher Einfluss von Ascorbat auf Mechanismen der Sauerstoff-Rezeption *in vivo* untersucht.

Hierzu fand ein genetisch modifiziertes Tiermodell mit Mäusen Verwendung, die eine biallelische Inaktivierung des L-Gulonolacton Oxidasegens (Gulo) aufweisen, das für ein Schlüsselenzym zur körpereigenen Synthese von Ascorbat kodiert. Folglich sind diese Tiere abhängig von der Aufnahme von Ascorbat mit der Nahrung. Im Vergleich zu Tieren, die Ascorbat mit dem Trinkwasser erhielten, zeigten $Gulo^{-/-}$ Mäuse, denen fünf Wochen lang eine Ascorbat-freie Diät gefüttert wurde, keine wesentlichen Änderungen in der Expression von HIF Zielgenen. Ausserdem konnten keine merklichen Unterschiede zwischen beiden Behandlungsgruppen in der Expression von EPO mRNA in der Niere und zirkulierendem EPO Protein im Plasma nach einer 24-stündigen hypoxischen Episode mit einer inspiratorischen Sauerstoffkonzentration von ca. 8% O_2 festgestellt werden. Unsere Daten legen nahe, dass eine Ascorbat-Defizienz keinen Einfluss auf die systemische Adaptation an Sauerstoffmangelzustände hat und die Rolle von Ascorbat im Organismus durch andere Antioxidantien kompensiert werden kann. Diese Hypothese findet zusätzlich Unterstützung in der Beobachtung, dass humane Zervixkarzinom-Zellen, die unter vollständig Ascorbat-freien Kulturbedingungen gehalten wurden, eine identische hypoxische HIF-1α Stablilisierung und transkriptionelle HIF Aktivität zeigten wie Zellen, die in Gegenwart von 50 μM Ascorbat wuchsen. Glutathion (GSH) ist das weitaus verbreitetste Antioxidant in menschlichen Zellen, weswegen wir den Einfluss von GSH auf die enzymatische Aktivität von PHDs *in vitro* untersucht haben. Tatsächlich wurden alle drei PHD Isoformen in dosisabhängiger Weise durch GSH aktiviert. Analog zur Behandlung mit Ascorbat zeigten humane Hepatomazellen in Gegenwart von GSH einen reduzierten HIF-1α Proteingehalt, wie auch die transkriptionelle Aktivität von Kobalt(II)-induziertem HIF vermindert war. Desweiteren veringerte Glutathion die durch Fenton-Reaktion bzw. Kobalt(II)- und Wasserstoffperoxid-vermittelte Proteinoxidation von rekombinantem PHD2 Enzym, wie durch das Messen der Proteinkarbonylierung bestimmt wurde. Interessanterweise konnte die Mutation des Cysteinrestes an Position 201 des Proteins zu Serin die basale Hydroxylierungsaktivität von PHD2 erhöhen und steigerte die Resistenz des rekombinanten Proteins gegenüber oxidativen Noxen. Dieser strukturell zugängliche

und konservierte Cysteinrest stellt daher einen möglichen Angriffspunkt zur antioxidativen Aktivierung von PHDs durch Vitamin C und Glutathion dar.

3. Summary

Oxygen sensing by prolyl-4-hydroxylases domain (PHD) proteins is involved in many physiological as well as pathophysiological processes in the human body. Hypoxia-inducible factors (HIFs) are the prime hydroxylation targets of PHDs and function as oxygen regulated transcription factors controlling the expression of more than 100 target genes. Genes involved in erythropoiesis (e.g. EPO), angiogenesis (e.g. VEGF) as well as glucose transport and metabolism (e.g. GLUT1, PDK1) are the most prominent examples of HIF-regulated genes. PHD activity is regulated primarily by the availability of oxygen but also other co-factors and co-substrates can affect their hydroxylation capacity. Using a reconstituted hydroxylation assay we attempted to investigate the dependency of all three PHDs isoforms on different types of small molecule activators and inhibitors. While addition of iron (II), 2-oxoglutarate and ascorbate was enhancing PHD activity in a dose-dependent manner, other compounds such as succinate or polyphenolic antioxidants were inhibiting hydroxylation activity of all three PHDs. Transition metals (cobalt (II), zinc (II)), metal chelators (DFX, EDTA) and reactive oxygen species (hydrogen peroxide) were efficient inhibitors of PHD activity. The mechanisms of action of these compounds could be explained at least partially by depletion of one of the co-factors necessary for the hydroxylation reaction. We could show that copper (II), but not cobalt (II) and iron (II) catalysed oxidation of ascorbate to dehydroascorbate, which is incapable to activate PHDs. Ascorbate deficiency is known to cause a systemic disorder called scurvy, resulting from insufficient hydroxylation of collagen by collagen prolyl-4-hydroxlases in connective tissues. PHDs belong to the same subclasses of enzymes as collagen prolyl-4-hydroxylases, therefore we attempted to investigate whether ascorbate deficiency can affect oxygen sensing mechanisms *in vivo*. We used a knock-out mouse model lacking the key enzyme involved in ascorbic acid synthesis, Gulono-1,4-lactone oxidase (Gulo) and as a consequence the animals depend on ascorbate supplementation in the diet. When $Gulo^{-/-}$ mice were kept for 5 weeks at an ascorbate-free diet, expression of HIF-target genes was unchanged if compared to control animals receiving ascorbate with the drinking water. Moreover, when $Gulo^{-/-}$ mice were exposed to inspiratory hypoxia (8% oxygen for 24 hours) after 5 weeks of ascorbate depletion, no significant changes to the ascorbate fed control group between Epo mRNA in the kidney and EPO plasma protein levels were observed. These data

suggest, that ascorbate deficiency might be compensated by other antioxidants *in vivo*. In support with this notion, HeLa cells grown in the absence of ascorbate showed equal hypoxic stabilization of HIF-1α protein and HIF activity as cells supplemented with 50 μM ascorbate. The most abundant intracellular antioxidant in human cells is glutathione (GSH). Therefore we tested its influence on PHDs activity *in vitro*. All three PHDs were robustly activated by GSH in a dose-dependent manner. Similarly to ascorbate, GSH could reduced HIF1-α protein levels and HIF transcriptional activity in cobalt (II) treated hepatoma cells. Glutathione protected recombinant PHD2 enzyme from oxidation by Fenton reaction and cobalt (II) as measured by carbonylation of the PHD2 protein. Interestingly, a C201S mutation in PHD2 increased basal hydroxylation rates of the enzyme and conferred resistance to oxidative damage *in vitro*, suggesting that this structurally conserved and surface accessible cysteine residue could be a target of antioxidative protection of PHDs by vitamin C and glutathione.

Table of contents:

4. Introduction

Oxygen (O_2) was first discovered in 1772 by Carl Wilhelm Scheele, the Swedish chemist (Scheele, 1777). Shortly after this discovery, the British clergymen Joseph Priestley and the French scientist Antoine Lavoisier succeeded to purify the "air responsible for combustion", which Lavoisier named "oxygen", respectively, and thus together with Carl Wilhelm Scheele are credited for the discovery of oxygen (Priestley, 1775). Since that time, the physical and chemical properties of oxygen were fully characterized. The function of oxygen in processes such as oxidative phosphorylation and photosynthesis further emphasized its important role in physiology and cell metabolism. Since then, oxygen has become the "life" gas, which drives many physiological processes in the living organisms. Interestingly, oxygen starvation, termed "hypoxia" is occurring at physiological and pathophysiological conditions. Such conditions include exposure to high altitude, where O_2 partial pressures (pO_2) are much lower than at sea level. Moreover, organs and tissues in the human body require constant oxygen supply to maintain their function and balanced energy homeostasis, and as such are affected by insufficient oxygen supply by mechanisms including ischemia or inflammation. It was first reported in the 19th century by Denis Jourdanet and Paul Bert, that the concentration of red blood cells in humans and animals depends on the O_2 supply to the tissue (Bert, 1878; Bert, 1882; Jelkmann, 2007). With the discovery of a factor, which was later characterized as a polypeptide hormone called erythropoietin, it was elucidated that this hormone regulates red blood cell proliferation and differentiation (Erslev, 1953; P Carnot, 1906). However, understanding of the molecular mechanisms that underlie effects of hypoxia on erythropoiesis remained unresolved until the end of the 20th century.

4.1 The hypoxia-inducible factor (HIF)

4.1.1 Discovery of HIF

The mechanism of induction of erythropoietin and enhanced red blood cell production upon oxygen deprivation was a long discussed phenomenon in the scientific community. The mechanism was later elucidated with the discovery that a hypoxia-inducible nuclear factor was binding to a regulatory enhancer element located 3´-to

the human erythropoietin gene (Semenza et al., 1991). The hypoxia inducible factor, named HIF, was later described as a transcription factor induced by hypoxia via *de novo* protein synthesis and binding to the human erythropoietin gene enhancer, causing transcriptional induction of this gene (Semenza and Wang, 1992). In 1995, the same group purified and characterized HIF-1 (Wang and Semenza, 1995). HIF was also described in other mammalian cells cultured under reduced oxygen tension, necessary for transcriptional activation of erythropoietin and other genes, providing evidence for a universal oxygen-sensing mechanism (Maxwell et al., 1993).

4.1.2 Composition of HIF complexes

Hypoxia-inducible factors are composed of two subunits: a constitutively active HIF-β subunit and the oxygen-regulated HIF-α subunit, which form the transcriptionally active heterodimer (Wang et al., 1995). Both subunits are members of the basic helix-loop-helix/Per-Arnt-Sim (bHLH/PAS) family of transcription factors (Jiang et al., 1996). HIF-α subunits exist in three isoforms: HIF-1α, HIF-2α and HIF-3α (Figure 1). The first two (HIF-1α and HIF-2α) form heterodimers with HIF-1β resulting in transcriptionally active hypoxia-inducible factors (HIF). The third isoform, HIF-3α does not form functional transcription factors, but is known to inhibit HIF-1 and HIF-2 mediated transcription (Lisy et al., 2008; Gu et al., 1998). Moreover, an alternative splicing isoform of HIF-3α, termed inhibitory PAS domain protein (IPAS), is known to be hypoxia-inducible and provides a negative feedback loop suppressing HIF activity (Makino et al., 2001). The N-terminal part of HIFα consist of a bHLH and PAS domain, which are responsible for DNA binding and dimerization with the HIF-β subunit. The HLH motifs of both subunits (HIF-1α and HIF-1β) contact the DNA. An oxygen-dependent degradation domain (ODDD) contains two distinct proline residues, which are the target for hydroxylation by prolyl-4-hydroxylases, thus regulating HIF-α protein stability (for detailed mechanism of HIF-α stability control, see below). In the C-terminal portion of HIF-α two transactivation domains are located, which recruit transcriptional coactivators to form an inititation complex (Pugh et al., 1997; Lisy et al., 2008). HIF-1α and HIF-2α contain a C-terminal transactivation domain (CAD) and more centrally located a N-terminal transactivation domain (NAD). Notably, transcriptional activity of the CAD domain is also regulated in an oxygen-dependent manner and an inhibitory domain (ID), located between the

two transactivation domains, is required for normoxic repression of the CAD (Lisy et al., 2008).

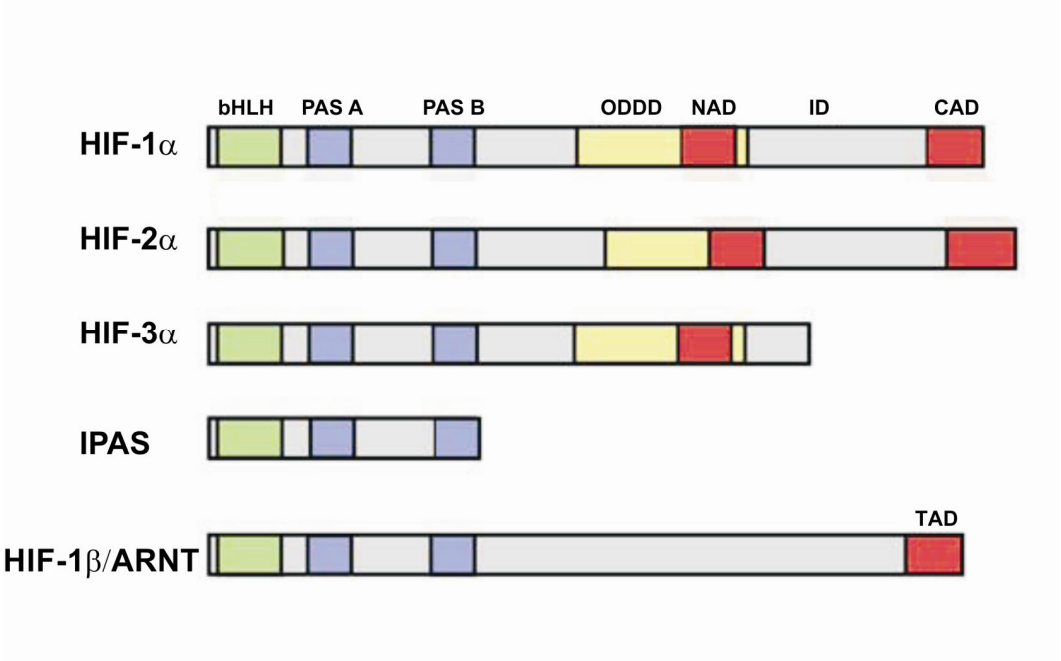

Fig. 1. Composition of HIF-α, IPAS and HIF-1β subunits (adapted from (Lisy et al., 2008)).

4.1.3 Function of HIF

The primary function of HIF is to regulate cellular responses to lowered oxygen concentrations. HIF acts as a transcription factor and regulates the expression of genes involved in adaptation to low oxygen concentrations. HIF binds to conserved hypoxia response elements (HREs) often found in the promoter (though not exclusicely) region of its target genes. The actual HIF binding site is a conserved sequence of 5'-[A/G]CGTG-3' (Wenger et al., 2005). So far, around 100 HIF target genes have been identified, which regulate adaptation to hypoxia on both, cellular and systemic levels (Wenger et al., 2005). Amongst others, these genes include vascular endothelial growth factor, glycolytic enzymes, glucose transporters, heme oxygenase-1, inducible nitric oxide synthase and transferrin (Forsythe et al., 1996; Semenza et al., 1994; Lee et al., 1997; Melillo et al., 1997; Rolfs et al., 1997).

4.1.4 Tissue distribution of HIF isoforms

HIF isoforms display tissue-dependent expression patterns. The HIF-1α isoform is ubiquitously expressed in all cells of the body and shows tissue-specific regulation

(oxygen-dependent protein stability regulation) under hypoxic conditions (Stroka et al., 2001). Similarly, the HIF-1β subunit shows ubiquitous expression. HIF-2α, initially known as EPAS1 (endothelial PAS protein 1) was first identified in endothelial cells of embryonic and adult mice as well as in highly vascularized tissues (Tian et al., 1997). Later, HIF-2α expression was described in other cell types and organs (Wiesener et al., 2003). The third isoform, HIF-3α is expressed in adult thymus, lung, brain, heart, and kidney (Gu et al., 1998).

4.1.4.1 Genetic ablation of HIF

Knock-out of HIF-1α in mice leads to embryonic lethality by day 11 due to cardiovascular malformation and open neural tube defects (Iyer et al., 1998; Kotch et al., 1999). HIF-2α knock-out mice show different phenotypes, varying from embryonic death between 12.5-16.5 days after gestation due to bradycardia caused by impaired catecholamine homeostasis (Tian et al., 1998) or neonatal death due to respiratory distress syndrome (Compernolle et al., 2002). Using a different mouse strain (129S6/SvEvTac) Scortegagna *et al.* reported viable HIF-2α knock-out mice which displayed multiple organ pathologies, biochemical abnormalities and changes in gene expression (Scortegagna et al., 2003). Interestingly, some of these phenotypes could be reversed by treatment of HIF-2α knock-out mice with SOD (superoxide dismutase) mimetic, suggesting involvement of HIF-2α in antioxidative defense and mitochondrial homeostasis. Also HIF-1β knock-out mice die in the embryonic stage at day 10.5, presumably due to placental, vascular and haematopoietic defects (Kozak et al., 1997).

4.1.5 Regulation of HIF

HIF expression and protein stability is regulated in both, oxygen-dependent and independent manners. Oxygen-dependent regulation is the primary mechanism by which HIF serves as a regulator of the transcriptional response to lowered oxygen concentrations. Moreover, HIF transcription and translation can be regulated by several other factors, which contribute to the primary mechanism of HIF regulation by oxygen-deficiency.

4.1.5.1 Oxygen-dependent regulation of HIF protein stability

HIF-α subunits are constitutively expressed and translated in cells. However, the half-life of the protein under normoxic conditions is less than 5 minutes (Lisy et al., 2008; Wang et al., 1995; Yu et al., 1998). The rapid degradation of HIF-α under normoxic conditions is mediated by enzymes called HIF prolyl-4-hydroxylases. These enzymes require molecular oxygen and other co-factors to catalyze hydroxylation of two distinct proline residues within HIF-α (Huang et al., 2002; Ivan et al., 2001; Jaakkola et al., 2001). Human HIF-1α is hydroxylated at Pro402 and Pro564 and HIF-2α at Pro405 and Pro531, respectively. Hydroxylated HIF-α is recognized by the von Hippel-Lindau - elongin B - elongin C ubiquitin E3 ligase complex and targeted for proteosomal degradation. When oxygen concentration is reduced, HIF prolyl-4-hydroxylases are inhibited, HIF-α is stabilized and translocates to the nucleus. There it heterodimerizes with the constitutively expressed HIF-β subunit and the functional HIF complex regulates the expression of its target genes. Moreover, another hydroxylase, factor inhibiting HIF (FIH) is responsible for hydroxylation of Asn803 within CAD of human HIF-1α and Asn851 of HIF-2α, which inhibits recruitment of the transcriptional co-activators p300/CBP (Hewitson et al., 2002; Lando et al., 2002; Mahon et al., 2001; Nagel et al., 2010).

4.1.5.2 Oxygen–independent regulation of HIF transactivation

In addition to oxygen-dependent hydroxylation of proline and asparagine residues, HIF-α can also undergo other posttranslational modifications. HIF-α has been reported to be phopshorylated at Thr796 (Gradin et al., 2002). It has been also reported, that phosphorylation of serine residues (641-643) by MAPK within HIF-1α promotes its transcriptional activity (Mylonis et al., 2006; Richard et al., 1999; Sang et al., 2003). S-nitrosylation of Cys800 was shown to increase HIF transcriptional activity via enhancement of CAD-CBP/p300 interaction (Yasinska and Sumbayev, 2003). SUMOylation of HIF-1α has been reported, however the effect of this modification is unclear, giving contradictory effects on its transactivation activity (Berta et al., 2007; Carbia-Nagashima et al., 2007). Besides above mentioned modifications, HIF has been shown to be regulated by several growth factors, cytokines and hormones such as Insulin, IGF-2, TNFα, IL-1β, PDGF and thrombin (Dery et al., 2005; Stiehl et al., 2002). Also inhibitors of heat shock protein 90 (HSP90) have been shown to promote HIFα degradation (Ibrahim et al., 2005; Isaacs

et al., 2002). Unlike oxygen-dependent regulation, hypoxia-independent regulation of HIF is based on an increase in HIF translation but not in protein stabilization (Dery et al., 2005). Recent reports also showed an increase in HIF-1α mRNA expression upon stimulation with reactive oxygen species (ROS), which is presumably mediated via NFκB (Bonello et al., 2007; Belaiba et al, 2007; Frede et al., 2006).

4.2 HIF prolyl-4-hydroxylases (PHDs)

4.2.1 Discovery of PHDs

Prolyl-hydroxylation of HIFα subunits was first described in 2001 (Bruick and McKnight, 2001; Epstein et al., 2001; Ivan et al., 2002; Ivan et al., 2001; Jaakkola et al., 2001; Yu et al., 2001). It was observed that hydroxylation of HIF-α triggers its recognition by the pVHL – elongin B – elongin C complex and subsequent degradation. Later findings showed that HIF-α contains two prolines residues, which are both targets for hydroxylation by prolyl-4-hydroxylases, and that both residues can independently interact with pVHL complex (Lando et al., 2002; Masson et al., 2001).

4.2.2 Isomers of HIF-PHDs

So far three isoforms of HIF-prolyl-4-hydroxylases have been described in humans, namely PHD1, PHD2 and PHD3. These proteins are encoded by human *EGLN2, EGLN1* and *EGLN3* genes, respectively. The proteins differ in size, intracellular localization and tissue distribution (Wenger et al., 2009). Under experimental *in vitro* conditions, preferences of respective isoforms to the two target proline substrates within the HIF-α molecule have been observed (Appelhoff et al., 2004; Chan et al., 2005). A new putative prolyl-4-hydroxylase was identified in the endoplasmic reticulum, which, when overexpressed can suppress HIF protein levels activity similarly to PHDs (Koivunen et al., 2007; Oehme et al., 2002). However, further studies must be performed to elucidate the physiological function of this isoform. *C. elegans* and *D. melanogaster* have a single PHD isoform called Egl9 and Fatiga, respectively (Kaelin and Ratcliffe, 2008). All three human and HIF-prolyl-4-hydroxylases have a high sequence similarity in the catalytic domain as shown on Figure 2.

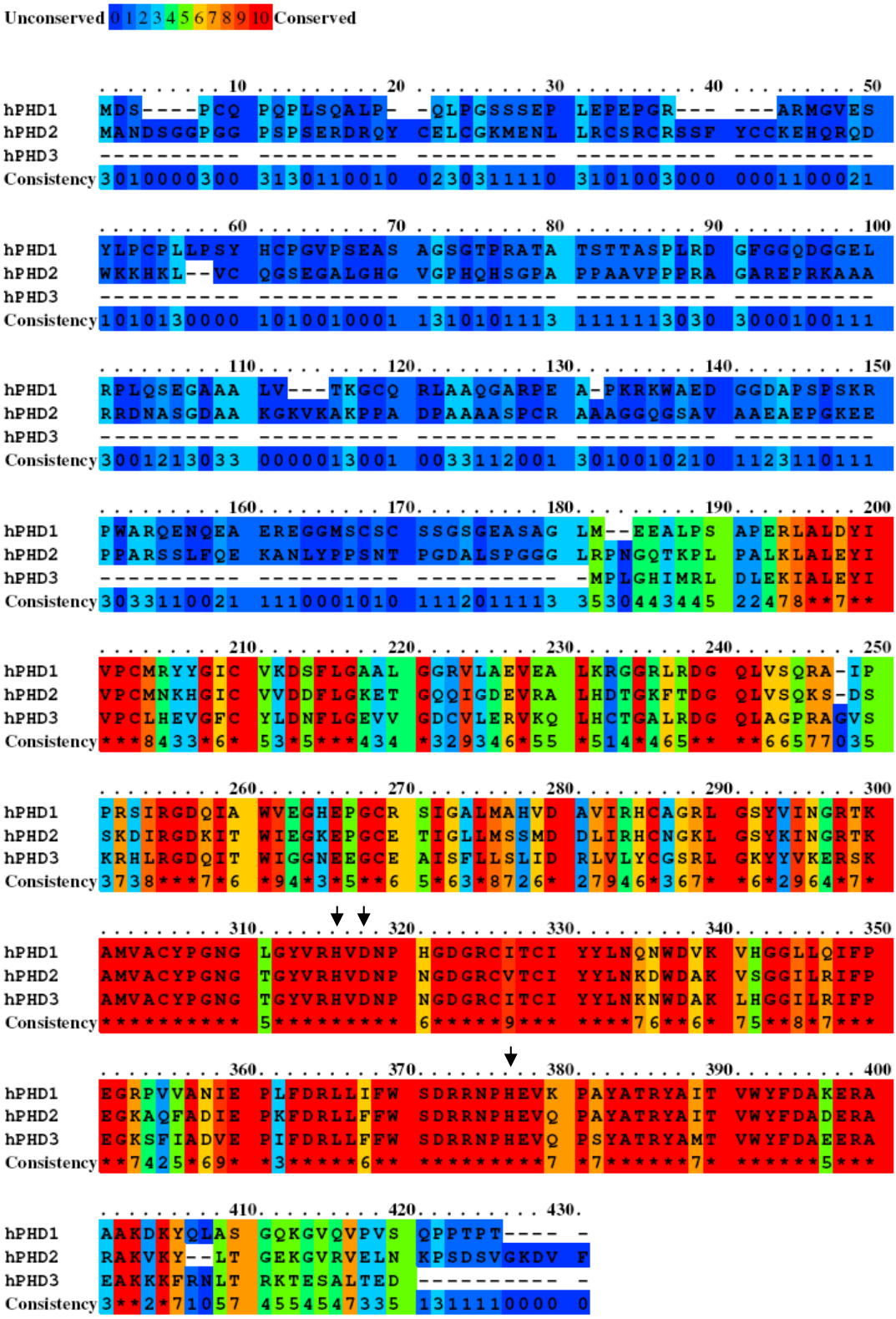

Fig. 2. Sequence alignment of three human HIF-PHDs. The scoring scheme works from 0 for the least conserved alignment position, up to 10 for the most conserved alignment position (alignment performed with the help of PRALINE program, ↓ indicates catalytic triade involved in iron coordination).

4.2.3 Cellular localization and tissue pattern of PHDs expression

HIF-PHDs show differences in both sub-cellular and tissue localization. Cellular localization of HIF-PHDs is somewhat controversy because of contradictory results between different groups. Immunofluorescence of exogenously expressed EGFP-PHDs in U2OS cells revealed that PHD1 is mainly localized in the nucleus, PHD2 and FIH are most abundant in the cytoplasm and PHD3 is distributed between both compartments (Metzen et al., 2003). However, another study showed that all three isoforms are located mainly in the cytoplasmatic compartment (Soilleux et al., 2005). These discrepancies might be explained by different methods used in the detection of PHDs. In the first study confocal fluorescence microscopy (detecting EGFP-PHDs) was used versus monoclonal antibodies raised against PHDs used in the second study. Moreover, nuclear localization of PHD2 have been reported in two independent studies suggesting a new regulatory function for PHD2 in the nuclei (Berchner-Pfannschmidt et al., 2008; Ozer et al., 2005). Among different tissues, the most abundant is PHD2, which is ubiquitously expressed in most tissues and is responsible for normoxic hydroxylation of HIFs (Berra et al., 2003) (Appelhoff et al., 2004; Stiehl et al., 2006). PHD1 shows highest expression in testis and PHD3 in the heart (Oehme et al., 2002; Soilleux et al., 2005). Moreover, PHD2 and PHD3 have been characterized as HIF target genes and their expression can be regulated by HIF, providing a negative feedback loop mechanism (Ginouves et al., 2008; Metzen et al., 2005; Minamishima et al., 2009; Pescador et al., 2005; Stiehl et al., 2006).

4.2.3.1 Genetic ablation of PHDs

Genetic ablation of only PHD2 (but not PHD1 and PHD3) leads to embryonic lethality (Takeda et al., 2006). Lethality of PHD2 knock-out mice occurs after embryonic day 12.5 due to placental and heart defects (Takeda et al., 2006). Somatic inactivation of PHD2 results in increased erythropoiesis and angiogenesis via HIF stabilization and induction of erythropoietin and vascular endothelial growth factor (Takeda et al., 2007). Interestingly, combined knock-out of both PHD1 and PHD3 shows a slight increase in hematocrit, hemoglobin concentration and red blood cell counts which supposedly resulted from increased EPO expression in the liver of adult mice (Takeda et al., 2008). PHD1 knock-out mice are viable, however their skeletal muscles show lower oxygen consumption and are protected against ischemic injury (Aragones et al., 2008). A similar protective effect of loss of PHD1 has been recently

reported also for the liver (Schneider et al., 2010). PHD3 knockout mice are viable but show abnormal sympathoadrenal development demonstrated by reduced adrenal medullary secretory capacity, sympathoadrenal responses and systemic hypotension (Bishop et al., 2008).

4.2.4 Enzymatic activity of PHDs

HIF prolyl-4-hydroxylases belong to the larger superfamily of 2-oxoglutarate and iron (II) dependent di-oxygenases. These enzymes use molecular di-oxygen and 2-oxoglutarate as co-substrates to hydroxylate target prolyl-residues within HIF-α. Iron (II) is incorporated into the active site of these enzymes and is bound by the catalytic triade (His313, Asp315 and His374 for PHD2) (McDonough et al., 2006). In the reaction catalyzed by PHDs, hydroxylation of the proline substrate is coupled to oxidative decarboxylation of 2-oxoglutarate in a stoichiometrical manner to give succinate and carbon dioxide as co-products (Chowdhury et al., 2009). One of the oxygen atoms is incorporated into succinate and the other into the hydroxyl-group of the proline substrate (Myllyharju, 2008). Iron (II), which binds the proline substrate and the oxygen molecule, undergoes oxidation with Fe (III) and Fe (IV) as intermediate states. The role of ascorbate in this reaction has not been fully explained, however it is proposed that ascorbate maintains iron in the active site of the PHDs in the reduced (ferrous) state. Moreover, in the absence of the proline substrate, prolyl-4-hydroxylases can catalyze an uncoupled reaction, in which 2-oxoglutarate is decarboxylated to succinate. It has been shown for the related collagen prolyl-4-hydroxylases that ascorbate is consumed stoichiometrically in uncoupled reaction cycles and can serve as an alternative oxygen acceptor in the absence of the proline substrate (Myllyharju, 2008; Myllylä et al., 1984). However, if uncoupled reaction cycles also occur with HIF prolyl-4-hydroxylases is not known and the actual role of ascorbate in the oxygen sensing prolyl hydroxylation is rather speculative.

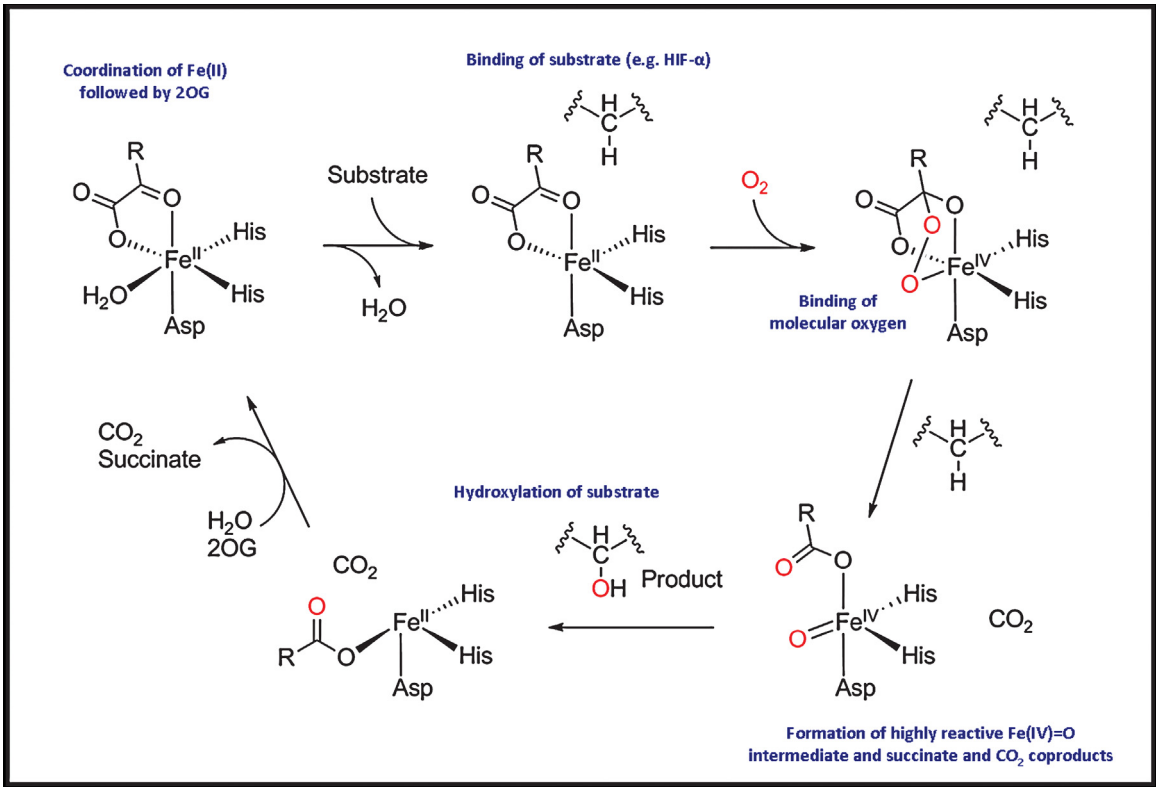

Fig. 3. Schematic cycle of HIF proline hydroxylation by PHDs (Nagel et al., 2010).

4.2.4.1 Homology to collagen-prolyl-4-hydroxylases.

HIF prolyl-4-hydroxylases belong to the same class of enzymes as collagen prolyl-4-hydroxylases (EC 1.14.11.2). However, the substrate for collagen prolyl-4-hydroxylases is a proline within collagen molecules where the role of hydroxyproline is to thermally stabilize the triple structure of collagen helixes. Both HIF and collagen prolyl-4-hydroxylases require oxygen, 2-oxoglutarate, iron (II) and/or ascorbate for activity, but they differ in *Km* values for these compounds. Structurally, collagen prolyl-4-hydroxylases are formed as α2β2 tetramers while PHDs presumably form α monomers in solution (known at least for PHD2), (McDonough et al., 2006; Myllyharju, 2008). Interestingly, HIF-PHD1-3 share 42-59% sequence identity to each other but only little sequence similarity to collagen prolyl-4-hydroxylases (Myllyharju, 2008) (Figure 4). However, the critical residues involved in binding of iron (II) and 2-oxoglutarate are conserved between both subclasses. Collagen prolyl-4-hydroxylases require only a X-Pro-Gly tripeptide as recognition site for hydroxylation, while HIF-PHDs recognize the longer sequence Leu-X-X-Leu-Ala-Pro

(Myllyharju, 2008). Accordingly, Pro 402 and Pro 564 of HIF-1α are not hydroxylated by collagen prolyl-4-hydroxylases (Bruick and McKnight, 2001).

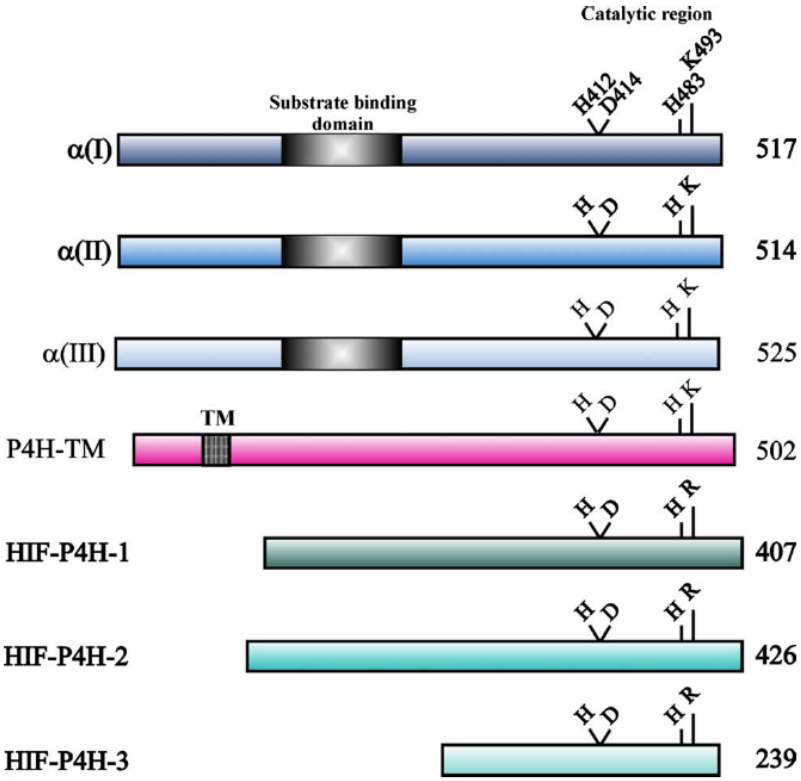

Fig. 4. Comparison of the human Collagen (I), (II) and (III) α subunits and transmembrane P4H with human PHD1-3 (Myllyharju, 2008). H-histidine, D-aspartic acid, K-lysine.

4.2.4.2 Co-factors for HIF prolyl-4-hydroxylases

Availability of oxygen, 2-oxoglutarate, iron (II) and/or ascorbate influences the hydroxylation capacity of PHDs. Therefore, changes in the concentration of these compounds can regulate PHDs activity and thus HIF protein abundance:

Oxygen

All three PHDs have a rather low affinity for oxygen with a *Km* of 230-250 μM, which is slightly above the atmospheric oxygen concentration (around 200 μM) (Hirsilä et al., 2003). This feature of HIF prolyl-4-hydroxylases enables them to respond to even small changes in oxygen concentration and activate the mechanism by which HIF can mediate responses to hypoxia. In comparison, collagen prolyl-4-hydroxylases have a roughly six-fold lower *Km* for oxygen (40 μM). Interestingly, FIH has also a lower *Km* for oxygen (90 μM) than PHDs, suggesting that its function is preserved even at reduced oxygen concentrations where HIF-α protein is already stabilized. However, the specific *Km* value of these enzymes can vary, depending on the enzyme purity and source (insect cell, bacteria) and substrate peptide length used in the study (Kaelin and Ratcliffe, 2008).

2-oxoglutarate

2-oxoglutarate is one of the intermediates of the Krebs cycle, thus any change in oxidative cell metabolism principally could affect its availability for PHDs. HIF-PHDs 1-3 have *Km* values for 2-oxoglutarate around 60 μM, which is roughly 3-fold higher than those reported for collagen prolyl-4-hydroxylase (20 μM) (Hirsilä et al., 2003). However, it has been shown, that PHD2 co-purifies with 2-oxoglutarate with a binding constant <<2 μM, suggesting that 2-oxoglutarate abundance is not a major physiological regulator of the HIF hydroxylation reaction (McNeill et al., 2005).

Iron (II)

The third compound necessary for the activity of PHDs is iron (II). The *Km* values estimated for HIF-PHDs were 30 nM for PHD1 and PHD2 or 100 nM for PHD3, respectively (Hirsila et al., 2005). Somewhat surprisingly, PHD2 co-purifies at oxic conditions as a complex with ferrous iron, even in the absence of ascorbate, suggesting that the enzyme itself provides an anti-oxidative scaffold for iron. The iron

binding constant for PHD2 was estimated as <<1 μM (McNeill et al., 2005). This high affinity of PHD2 for iron (II) and 2-oxoglutarate is unusual among other studied prolyl-4-hydroxylases (Chowdhury et al., 2009).

Ascorbate

Historically, ascorbate was described as an essential co-factor for the activity of collagen prolyl-4-hydroxylases. Ascorbate deficiency is known to cause a disease called scurvy, which symptoms, amongst others, include bleeding gums and internal hemorrhages resulting from insufficient hydroxylation of collagen triple helixes building up connective tissues (Linster and Van Schaftingen, 2007). However, HIF-PHDs have roughly 2-fold lower *Km* values (140-180 μM) for ascorbate than collagen prolyl-4-hydroxylases (300-370 μM) and there is no evidence in the literature that lack of ascorbate affects oxygen sensing *in vivo* (Hirsilä et al, 2003; Nagel et al., 2010). Of note, ascorbate does not co-purify with PHD2 like 2-oxoglutarate and iron (II) (McNeill et al., 2005). The role of ascorbate in the reaction catalyzed by HIF prolyl-4-hydroxylases is still not clear. As mentioned earlier, it is postulated, that ascorbate reduces the iron intermediate (IV) to its active (II) form, however it requires further investigation in the context of HIF-prolyl-4-hydroxylases.

Effect of the peptide length on the activity of PHDs

It was reported that PHDs (at least known for PHD1 and PHD2) hydroxylate Pro402 within human HIF-1α much less effectively than Pro564 (Hirsilä et al., 2003; Villar et al., 2007). This difference cannot be explained by the sequence differences surrounding the hydroxyl-acceptor proline residue (Hirsilä et al., 2003). In contrary to collagen prolyl-4-hydroxylases, none of the PHDs is able to hydroxylate short peptides between 3-7 aminoacids *in vitro* (Hirsilä et al., 2003). The *Km* value estimated for a 19-residue peptide corresponding to the C-terminal HIF-1α hydroxylation site was around 5-10 μM (Hirsilä et al., 2003). Shortening of the peptide at the C-terminus had an inhibitory effect on PHD1 and PHD2 and a smaller effect on PHD3. Peptides shorter than 11 residues were not hydroxylated by any of the isoforms (Hirsilä et al., 2003).

4.2.4.3 Regulation of PHDs activity by small molecules

Reactive oxygen species

The inhibitory effect of reactive oxygen species (ROS) on PHDs activity is not fully understood. ROS can either directly oxidize amino acid residues within the enzyme or indirectly deplete or inactivate one of the essential co-factors by oxidation (iron (II), ascorbate). There have been several reports showing that hypoxic cells generate higher levels of ROS, which could inhibit PHD activity. Moreover, cells lacking mitochondrial DNA have been reported to not respond to hypoxia by activating HIF-target gene expression, suggesting involvement of these organelles in oxygen sensing (Chandel et al., 1998). Of note, these hypotheses were not confirmed by other independent studies, which showed that oxygen sensing does not require functional mitochondria (Srinivas et al., 2001; Vaux et al., 2001). An example of indirect co-factor depletion was shown in JunD knock-out cells, which have increased amounts of ROS and PHDs activity is inhibited by reduced amount of available iron (II). Consequently, these cells activate HIF and HIF-target genes (Gerald et al., 2004).

Nitric oxide

Similarly to ROS, nitric oxide (NO) has been reported to modulate HIF stability, most likely due to inhibition of PHDs. It has been shown that nitric oxide can contribute to responses to hypoxia in *D. melanogaster* (Wingrove and O'Farrell, 1999). Interestingly, eNOS (endothelial NOS) is a HIF target gene, which could provide an additional feedback loop to oxygen sensing via HIF-PHDs (Kaelin and Ratcliffe, 2008). However, opposing effects of NO on HIF have been reported. It was shown that NO can induce HIF stabilization and target gene expression (Kimura et al., 2001). Contrarily, NO was shown to have bi-modal effects on HIF-1α protein accumulation: In a first instance, NO administration inhibits PHD activity leading to a rapid induction of HIF-1α protein. With some delay, HIF-dependent *de novo* PHD2 protein synthesis finally destabilizes HIF-1α, thus counteracting the initial NO response. (Berchner-Pfannschmidt et al., 2007). A similar model of bi-phasic responses to notoriously instable intermediates as ROS is also attractive to explain the many controversial results reported for this molecule group with respect to the oxygen sensing pathway.

Antioxidants

As ascorbate is required for full activity of HIF prolyl-4-hydroxylases *in vitro*, it was suggested that their activity is dependent on the presence of antioxidative agents. Whether the function of ascorbate is specific or can be replaced by other antioxidants is still unclear. A tumor-suppressive role of ascorbate - at least partially mediated by the HIF/PHD pathway - has been demonstrated *in vivo* (Gao et al., 2007). In this study, xenografted tumors grown in mice treated with ascorbate (5g/l) were significantly smaller than those of non-treated animals. Authors attributed this effect to the presence of HIF-1α, because tumors overexpressing HIF-1α were more resistant to ascorbate treatment. The same effects were observed when mice were treated with N-acetyl cysteine (40 mM). N-acetyl cysteine is an antioxidant but can also increase the intracellular pool of glutathione, as it serves as a precursor for this compound. Moreover, it was reported by Knowles *et al*., that ascorbate can decrease HIF-1α levels in cancer cells that have been treated with cobalt chloride and DFX. Nevertheless, ascorbate had no effect on hypoxia-treated cells (Knowles et al., 2003). Ascorbate administered at physiological concentration (25 μM) reduced normoxic levels of HIF-1α and prevented its induction by growth factors (IGF-1 and insulin) in a PHD dependent manner (Knowles et al., 2003). Recently, it was shown that REDD1 knock-out cells have increased production of ROS and loss of REDD1 confered tumorigenicity to mouse embryonic fibroblasts, which was dependent on the presence of HIF-1α. Antioxidant treatment with ascorbate reduced HIF-1α protein levels and inhibited tumorigenic growth of REDD1$^{-/-}$ cells *in vivo* (Horak et al., 2010; Knowles et al., 2003). Interestingly, catalase is routinely added to *in vitro* hydroxylation assays, however the function of this enzyme in the reaction is unclear. It is possible that catalase (and other antioxidants) scavenges some reactive oxygen species generated during catalytic cycle, or it might simply take part in the reduction of iron (IV) to iron (II). Suprisingly, it has been shown, that other antioxidants, such as gallate or n-propyl gallate exert opposite effects on PHDs. For example it was reported, that gallate, but not n-propyl gallate, could inhibit PHDs and stabilize HIF (Tsukiyama et al., 2006). This effect was explained by binding of the carboxyl group of gallate to Arg383 of PHD2 and chelation of iron (II) within the catalytic domain of PHD2. This binding would be inhibited by the n-propyl group of n-propyl gallate. Similarly, another plant-derived flavonoid, quercetin has inhibiting activity on PHD2 (Dao et al., 2009). Such

contradictory effects of different antioxidants could suggest different mechanisms by which these compounds activate/inhibit PHDs and act on HIF.

Transition metals

Transition metals and metal chelators have been implicated in the regulation of the HIF pathway. It has been shown that cobalt, nickel and zinc inhibit all three PHDs. Mechanistically, transition metals a thought to act either by replacement or oxidation of PHD bound iron (II). Alternatively, oxidation of ascorbate to dehydroascorbate catalyzed by transition metals has been proposed to activate the HIF pathway (Hirsilä et al., 2003; Salnikow et al., 2004). Another explanation for the inhibitory mechanism of cobalt could be direct binding to HIF-1α and therefore an inhibition of HIF-1α protein degradation by the VHL ubiquiutin ligase complex (Yuan et al., 2003). In comparison to collagen prolyl-4-hydroxylases and FIH, most metals were less effective inhibitors of PHDs, in particular PHD2 (Hirsilä et al., 2003). Among other metals, cadmium inhibited all three PHDs, whereas magnesium and manganese were the least potent inhibitors with half maximal inhibitory concentrations (IC_{50}) ranging from 50 to1 mM (Hirsilä et al., 2003). Also cupric copper is a potent inhibitor of PHD activity, inducing HIF activity and target gene expression (Martin et al., 2005). This can be explained by the fact that copper is a potent catalyst of ascorbate oxidation by air (Nytko et al., 2007).

2-oxoglutarate analogs

Many 2-oxoglutarate analogs serve as potent inhibitors of PHD activity. Since binding of 2-oxoglutarate to the enzyme is required for normal turnover of the enzyme, these compounds block the reaction by binding to the 2-oxoglutarate pocket and inhibiting enzyme activity. N-Oxaloylglycine (NOG) and its cell-permeable analogon dimethyl-oxalylglycine (DMOG) are the widest used small molecule inhibitors of PHD activity (Nagel et al., 2010; Oehme et al., 2004). Moreover, other Krebs cycle intermediates, such as succinate, fumarate, malate and pyruvate, have been shown to inhibit the activity of PHDs *in vitro* or in cell culture (Koivunen et al., 2007; Nytko et al., 2007; Pan et al., 2007). Interestingly, germline mutations in fumarate hydratase (FH) and succinate dehydrogenase (SDH) predispose these individuals to different types of tumors, supposedly due to HIF-1α stabilization (Pollard et al., 2005; Pollard et al., 2007).

4.2.5 Regulation of PHDs expression

PHD2 and PHD3 transcription is regulated by HIF, as these genes contain functional HRE sequences in the promoter or first intronic region of their genes, respectively (Metzen et al., 2005; Pescador et al., 2005). This provides a negative feedback loop and enables cells/tissues to adapt to prolonged hypoxic exposure (Minamishima et al., 2009; Stiehl et al., 2006). Moreover, PHD1 gene expression was reported to be induced by estradiol (Appelhoff et al., 2004). PHD2 expression is regulated by TGF-β, which decreases PHD2 mRNA and protein levels and accordingly induces HIF-1 transcriptional activity (McMahon et al., 2006).

4.2.6 Other regulators of PHDs

It has been reported that PHDs might interact with other molecules that may affect their stability and/or activity. One of the first characterized interactors for PHD1 and PHD3 was the RING finger E3 ubiquitin ligase Siah2, which regulates PHD1 and PHD3 protein stability. Moreover, Siah2 knock-out mice show an impaired response to hypoxia with reduced levels of hemoglobin, emphasizing the physiological relevance of the Siah2-PHD3 interaction (Nakayama et al., 2004). Another interaction, which was characterized in the course of the current thesis (see contribution to publications) is FK506-binding protein 38 (FKBP38), which specifically binds PHD2 and regulates its protein stability (Barth et al., 2007). A list of characterized interactors of PHDs are summarized in Table 1 (adapted from (Wenger et al., 2009). Some of the interactors are putative hydroxylation targets for PHDs.

Interactor	PHD1	PHD2	PHD3	Reference
Siah2			+	(Nakayama et al., 2004)
FKBP38		+		(Barth et al., 2007)
TriC			+	(Masson et al., 2004)
OS-9		+	+	(Baek et al., 2005)
AKAP		+		(Choi et al., 2007)
Morg1			+	(Hopfer et al., 2006)
ING4		+		(Ozer et al., 2005)
IOP1		+		(Huang et al., 2007)
MAGE		+		(Aprelikova et al., 2009)
Cdr2	+			(Balamurugan et al., 2009)
ATF4*			+	(Koditz et al., 2007)
IKKβ*	+			(Cummins et al., 2006)
Myogenin*			+	(Fu et al., 2007)
KIFBβ*			+	(Schlisio et al., 2008)
Rbp1*	+	+		(Mikhaylova et al., 2008)

Table 1. Characterized interactors of PHD1-3, asterisks indicate putative hydroxylation targets of PHDs (based on (Wenger et al., 2009)).

4.2.7 Role of PHDs in physiology and pathophysiology

Because PHDs are important factors regulating HIF stability, they also play a role in physiological and pathophysiological aspects of disorders in which HIF is involved.

Mutations in PHD2 have been described in patients suffering from erythrocytosis and polycythemia (Percy et al., 2007; Percy et al., 2006). These mutations occur at residues Pro317, Arg371 and His374 and all of them are located in the catalytic domain of PHD2, causing inhibition of hydroxylation activity (Ladroue et al., 2008; Percy et al., 2007; Percy et al., 2006). This discovery emphasizes the important role of PHD2 in maintaining homeostasis of the erythropoietic pathway. Accordingly, diseases such as anemia could be potentially treated with PHDs inhibitors.

Because the HIF/PHD pathway is also involved in ischemia-reperfusion conferred injury, PHD inhibitors have been tested in ischemic preconditioning (IPC) which protects myocardial tissue from subsequent ischemia (Nagel et al.; 2010). Besides myocardial tissue damage by ischemia, ischemic stroke is another possible candidate disease that could be treated with PHDs inhibitors used for IPC (Harten et al., 2010). Beneficial effects of PHD inhibitors have been also implicated in inflammatory bowel disease, as DMOG has been shown to be protective in a murine model of colitis

(Cummins et al., 2008). Moreover, PHDs inhibitors could be potentially used in other diseases involving abnormal vascularization such as in retinopathy of prematurity or in placental disease (Nagel et al., 2010).

Recently, opposing effects describing the role of PHDs in the cancer have been reported. PHD2, as the main normoxic HIF-α regulator, was desribed to play a role in both, cancer progression or regression. On the one hand, PHD2 levels were shown to be decreased in human breast carcinomas samples which was accompanied by increased number of mature blood vessels in the tumor (Bordoli et al., 2010; Chan et al., 2009). This effect underlines an important role of PHD2 in tumor angiogenesis. However, PHD2 haplo-deficiency in tumor bearing mice was reported to increase vessel normalization, which is a quite opposite effect to the previous study (Mazzone et al., 2009). The discrepancies between both studies was explained by the effect of PHD2 deficiency in tumor cells itself, which results in an increased angiogenic response, while PHD2 deficiency in the host endothelium results rather in vessel normalization (Chan and Giaccia, 2010).

Besides the essential co-factors 2-OG, iron (II) and/or ascorbate, only few compounds are known to activate PHDs (Nagel et al., 2010). PHD activators could potentially inhibit tumor growth by destabilizing HIF-α, as was shown for ascorbate and N-acetyl cystein in a tumor allograft model (Gao et al., 2007). Use of ascorbate in anti-cancer therapy became famous already in the 1970's, when Linus Pauling claimed beneficial effects of this antioxidant in treatment of terminally ill cancer patients (Cameron and Pauling, 1976). Moreover, recent report showed for the fisrt time, that an aggressive tumor phenotype in endometrial cancer is associated with low ascorbate levels and increased HIF-1α activity (Kuiper et al., 2010). Another disorder, where PHDs activators could prove useful is hypoxia-induced pulmonary hypertension, since hypoxic stabilization of HIF is involved in pulmonary vasculature remodeling and causes high mortality among patients with lung diseases (Nagel et al., 2010).

In summary, PHDs activators and inhibitors are potential candidates for novel treatments for a number of diseases. Several studies report on small molecule library screens where compounds have been analysed for their potential to block or stimulate PHD activity in order to modify HIF levels in patients. The major obstacle however, is that the similarity between PHDs isoforms and other prolyl-4-hydroxylases may prevent specific targeting of PHDs, possibly leading to severe side effects when applied to the human body.

References:

Appelhoff RJ, Tian YM, Raval RR, Turley H, Harris AL, Pugh CW, Ratcliffe PJ, Gleadle JM (2004). Differential function of the prolyl hydroxylases PHD1, PHD2, and PHD3 in the regulation of hypoxia-inducible factor. J Biol Chem. 279(37): 38458-65. Epub 2004 Jul 7.

Aprelikova O, Pandolfi S, Tackett S, Ferreira M, Salnikow K, Ward Y, Risinger JI, Barrett JC, Niederhuber J (2009). Melanoma antigen-11 inhibits the hypoxia-inducible factor prolyl hydroxylase 2 and activates hypoxic response. Cancer Res. 69(2): 616-24.

Aragones J, Schneider M, Van Geyte K, Fraisl P, Dresselaers T, Mazzone M, Dirkx R, Zacchigna S, Lemieux H, Jeoung NH, Lambrechts D, Bishop T, Lafuste P, Diez-Juan A, Harten SK, Van Noten P, De Bock K, Willam C, Tjwa M, Grosfeld A, Navet R, Moons L, Vandendriessche T, Deroose C, Wijeyekoon B, Nuyts J, Jordan B, Silasi-Mansat R, Lupu F, Dewerchin M, Pugh C, Salmon P, Mortelmans L, Gallez B, Gorus F, Buyse J, Sluse F, Harris RA, Gnaiger E, Hespel P, Van Hecke P, Schuit F, Van Veldhoven P, Ratcliffe P, Baes M, Maxwell P, Carmeliet P (2008). Deficiency or inhibition of oxygen sensor Phd1 induces hypoxia tolerance by reprogramming basal metabolism. Nat Genet. 40(2): 170-80.

Baek JH, Mahon PC, Oh J, Kelly B, Krishnamachary B, Pearson M, Chan DA, Giaccia AJ, Semenza GL (2005). OS-9 interacts with hypoxia-inducible factor 1alpha and prolyl hydroxylases to promote oxygen-dependent degradation of HIF-1alpha. Mol Cell. 17(4): 503-12.

Balamurugan K, Luu VD, Kaufmann MR, Hofmann VS, Boysen G, Barth S, Bordoli MR, Stiehl DP, Moch H, Schraml P, Wenger RH, Camenisch G (2009). Onconeuronal cerebellar degeneration-related antigen, Cdr2, is strongly expressed in papillary renal cell carcinoma and leads to attenuated hypoxic response. Oncogene. 28(37): 3274-85.

Barth S, Nesper J, Hasgall PA, Wirthner R, Nytko KJ, Edlich F, Katschinski DM, Stiehl DP, Wenger RH, Camenisch G (2007). The peptidyl prolyl cis/trans isomerase FKBP38 determines hypoxia-inducible transcription factor prolyl-4-hydroxylase PHD2 protein stability. Mol Cell Biol. 27(10): 3758-68.

Belaiba RS, Bonello S, Zahringer C, Schmidt S, Hess J, Kietzmann T, Gorlach A (2007). Hypoxia up-regulates hypoxia-inducible factor-1alpha transcription by involving phosphatidylinositol 3-kinase and nuclear factor kappaB in pulmonary artery smooth muscle cells. Mol Biol Cell. 18(12): 4691-7.

Berchner-Pfannschmidt U, Tug S, Trinidad B, Oehme F, Yamac H, Wotzlaw C, Flamme I, Fandrey J (2008). Nuclear oxygen sensing: induction of endogenous prolyl-hydroxylase 2 activity by hypoxia and nitric oxide. J Biol Chem. 283(46): 31745-53.

Berchner-Pfannschmidt U, Yamac H, Trinidad B, Fandrey J (2007). Nitric oxide modulates oxygen sensing by hypoxia-inducible factor 1-dependent induction of prolyl hydroxylase 2. J Biol Chem. 282(3): 1788-96.

Berra E, Benizri E, Ginouvés A, Volmat V, Roux D, Pouysségur J (2003). HIF prolyl-hydroxylase 2 is the key oxygen sensor setting low steady-state levels of HIF-1α in normoxia. EMBO J 22(16): 4082-90.

Bert P (1878). Recherches de Physiologie Experimentale. Le Pression Barometrique.

Bert P (1882). Sur la richesse en hemoglobine du sang des animaux vivant sur les hauts lieux. C R Acad Sci Paris(94): 805-807.

Berta MA, Mazure N, Hattab M, Pouyssegur J, Brahimi-Horn MC (2007). SUMOylation of hypoxia-inducible factor-1alpha reduces its transcriptional activity. Biochem Biophys Res Commun. 360(3): 646-52.

Bishop T, Gallagher D, Pascual A, Lygate CA, de Bono JP, Nicholls LG, Ortega-Saenz P, Oster H, Wijeyekoon B, Sutherland AI, Grosfeld A, Aragones J, Schneider M, van Geyte K, Teixeira D, Diez-Juan A, Lopez-Barneo J, Channon KM, Maxwell PH, Pugh CW, Davies AM, Carmeliet P, Ratcliffe PJ (2008). Abnormal sympathoadrenal development and systemic hypotension in PHD3-/- mice. Mol Cell Biol. 28(10): 3386-400.

Bonello S, Zahringer C, BelAiba RS, Djordjevic T, Hess J, Michiels C, Kietzmann T, Gorlach A (2007). Reactive oxygen species activate the HIF-1alpha promoter via a functional NFkappaB site. Arterioscler Thromb Vasc Biol. 27(4): 755-61. Epub 2007 Feb 1.

Bordoli MR, Stiehl DP, Borsig L, Kristiansen G, Hausladen S, Schraml P, Wenger RH, Camenisch G Prolyl-4-hydroxylase PHD2- and hypoxia-inducible factor 2-dependent regulation of amphiregulin contributes to breast tumorigenesis. Oncogene 2010: 20.

Bruick RK, McKnight SL (2001). A conserved family of prolyl-4-hydroxylases that modify HIF. Science 294(5545): 1337-40.

Cameron E, Pauling L (1976). Supplemental ascorbate in the supportive treatment of cancer: Prolongation of survival times in terminal human cancer. Proc Natl Acad Sci U S A. 73(10): 3685-9.

Carbia-Nagashima A, Gerez J, Perez-Castro C, Paez-Pereda M, Silberstein S, Stalla GK, Holsboer F, Arzt E (2007). RSUME, a small RWD-containing protein, enhances SUMO conjugation and stabilizes HIF-1alpha during hypoxia. Cell. 131(2): 309-23.

Carnot P (1906). Sur l'activite hemopoietique du serum. Compt rend Acad d sc (143): 384.

Chan DA, Giaccia AJ (2010). PHD2 in tumour angiogenesis. Br J Cancer. 103(1): 1-5.

Chan DA, Kawahara TL, Sutphin PD, Chang HY, Chi JT, Giaccia AJ (2009). Tumor vasculature is regulated by PHD2-mediated angiogenesis and bone marrow-derived cell recruitment. Cancer Cell. 15(6): 527-38.

Chan DA, Sutphin PD, Yen SE, Giaccia AJ (2005). Coordinate regulation of the oxygen-dependent degradation domains of hypoxia-inducible factor 1 alpha. Mol Cell Biol. 25(15): 6415-26.

Chandel NS, Maltepe E, Goldwasser E, Mathieu CE, Simon MC, Schumacker PT (1998). Mitochondrial reactive oxygen species trigger hypoxia-induced transcription. Proc Natl Acad Sci U S A. 95(20): 11715-20.

Choi YK, Kim JH, Kim WJ, Lee HY, Park JA, Lee SW, Yoon DK, Kim HH, Chung H, Yu YS, Kim KW (2007). AKAP12 regulates human blood-retinal barrier formation by downregulation of hypoxia-inducible factor-1alpha. J Neurosci. 27(16): 4472-81.

Chowdhury R, McDonough MA, Mecinovic J, Loenarz C, Flashman E, Hewitson KS, Domene C, Schofield CJ (2009). Structural basis for binding of hypoxia-inducible factor to the oxygen-sensing prolyl hydroxylases. Structure 17(7): 981-9.

Compernolle V, Brusselmans K, Acker T, Hoet P, Tjwa M, Beck H, Plaisance S, Dor Y, Keshet E, Lupu F, Nemery B, Dewerchin M, Van Veldhoven P, Plate K, Moons L, Collen D, Carmeliet P (2002). Loss of HIF-2alpha and inhibition of

VEGF impair fetal lung maturation, whereas treatment with VEGF prevents fatal respiratory distress in premature mice. Nat Med. 8(7): 702-10.

Cummins EP, Berra E, Comerford KM, Ginouves A, Fitzgerald KT, Seeballuck F, Godson C, Nielsen JE, Moynagh P, Pouyssegur J, Taylor CT (2006). Prolyl hydroxylase-1 negatively regulates IkappaB kinase-beta, giving insight into hypoxia-induced NFkappaB activity. Proc Natl Acad Sci U S A. 103(48): 18154-9. Epub 2006 Nov 17.

Cummins EP, Seeballuck F, Keely SJ, Mangan NE, Callanan JJ, Fallon PG, Taylor CT (2008). The hydroxylase inhibitor dimethyloxalylglycine is protective in a murine model of colitis. Gastroenterology. 134(1): 156-65.

Dao JH, Kurzeja RJ, Morachis JM, Veith H, Lewis J, Yu V, Tegley CM, Tagari P (2009). Kinetic characterization and identification of a novel inhibitor of hypoxia-inducible factor prolyl hydroxylase 2 using a time-resolved fluorescence resonance energy transfer-based assay technology. Anal Biochem. 384(2): 213-23.

Dery MA, Michaud MD, Richard DE (2005). Hypoxia-inducible factor 1: regulation by hypoxic and non-hypoxic activators. Int J Biochem Cell Biol. 37(3): 535-40.

Epstein AC, Gleadle JM, McNeill LA, Hewitson KS, O'Rourke J, Mole DR, Mukherji M, Metzen E, Wilson MI, Dhanda A, Tian YM, Masson N, Hamilton DL, Jaakkola P, Barstead R, Hodgkin J, Maxwell PH, Pugh CW, Schofield CJ, Ratcliffe PJ (2001). C. elegans EGL-9 and mammalian homologs define a family of dioxygenases that regulate HIF by prolyl hydroxylation. Cell. 107(1): 43-54.

Erslev A (1953). Humoral regulation of red cell production. Blood. 8(4): 349-57.

Forsythe JA, Jiang BH, Iyer NV, Agani F, Leung SW, Koos RD, Semenza GL (1996). Activation of vascular endothelial growth factor gene transcription by hypoxia-inducible factor 1. Mol Cell Biol. 16(9): 4604-13.

Frede S, Stockmann C, Freitag P, Fandrey J (2006). Bacterial lipopolysaccharide induces HIF-1 activation in human monocytes via p44/42 MAPK and NF-kappaB. Biochem J. 396(3): 517-27.

Fu J, Menzies K, Freeman RS, Taubman MB (2007). EGLN3 prolyl hydroxylase regulates skeletal muscle differentiation and myogenin protein stability. J Biol Chem. 282(17): 12410-8.

Gao P, Zhang H, Dinavahi R, Li F, Xiang Y, Raman V, Bhujwalla ZM, Felsher DW, Cheng L, Pevsner J, Lee LA, Semenza GL, Dang CV (2007). HIF-dependent antitumorigenic effect of antioxidants in vivo. Cancer Cell 12(3): 230-8.

Gerald D, Berra E, Frapart YM, Chan DA, Giaccia AJ, Mansuy D, Pouysségur J, Yaniv M, Mechta-Grigoriou F (2004). JunD reduces tumor angiogenesis by protecting cells from oxidative stress. Cell. 118(6): 781-94.

Ginouves A, Ilc K, Macias N, Pouyssegur J, Berra E (2008). PHDs overactivation during chronic hypoxia "desensitizes" HIFalpha and protects cells from necrosis. Proc Natl Acad Sci U S A. 105(12): 4745-50.

Gradin K, Takasaki C, Fujii-Kuriyama Y, Sogawa K (2002). The transcriptional activation function of the HIF-like factor requires phosphorylation at a conserved threonine. J Biol Chem. 277(26): 23508-14.

Gu YZ, Moran SM, Hogenesch JB, Wartman L, Bradfield CA (1998). Molecular characterization and chromosomal localization of a third alpha-class hypoxia inducible factor subunit, HIF3alpha. Gene Expr. 7(3): 205-13.

Harten SK, Ashcroft M, Maxwell PH Prolyl hydroxylase domain inhibitors: a route to HIF activation and neuroprotection. Antioxid 12(4): 459-80.

Hewitson KS, McNeill LA, Riordan MV, Tian YM, Bullock AN, Welford RW, Elkins JM, Oldham NJ, Bhattacharya S, Gleadle JM, Ratcliffe PJ, Pugh CW, Schofield CJ (2002). Hypoxia-inducible factor (HIF) asparagine hydroxylase is identical to factor inhibiting HIF (FIH) and is related to the cupin structural family. J Biol Chem. 277(29): 26351-5.

Hirsilä M, Koivunen P, Günzler V, Kivirikko KI, Myllyharju J (2003). Characterization of the human prolyl 4-hydroxylases that modify the hypoxia-inducible factor. J Biol Chem 278(33): 30772-80.

Hirsila M, Koivunen P, Xu L, Seeley T, Kivirikko KI, Myllyharju J (2005). Effect of desferrioxamine and metals on the hydroxylases in the oxygen sensing pathway. Faseb J. 19(10): 1308-10.

Hopfer U, Hopfer H, Jablonski K, Stahl RA, Wolf G (2006). The novel WD-repeat protein Morg1 acts as a molecular scaffold for hypoxia-inducible factor prolyl hydroxylase 3 (PHD3). J Biol Chem. 281(13): 8645-55.

Horak P, Crawford AR, Vadysirisack DD, Nash ZM, DeYoung MP, Sgroi D, Ellisen LW (2010). Negative feedback control of HIF-1 through REDD1-regulated ROS suppresses tumorigenesis. Proc 107(10): 4675-80.

Huang J, Song D, Flores A, Zhao Q, Mooney SM, Shaw LM, Lee FS (2007). IOP1, a novel hydrogenase-like protein that modulates hypoxia-inducible factor-1alpha activity. Biochem J. 401(1): 341-52.

Huang J, Zhao Q, Mooney SM, Lee FS (2002). Sequence determinants in hypoxia-inducible factor-1alpha for hydroxylation by the prolyl hydroxylases PHD1, PHD2, and PHD3. J Biol Chem. 277(42): 39792-800.

Ibrahim NO, Hahn T, Franke C, Stiehl DP, Wirthner R, Wenger RH, Katschinski DM (2005). Induction of the hypoxia-inducible factor system by low levels of heat shock protein 90 inhibitors. Cancer Res. 65(23): 11094-100.

Isaacs JS, Jung YJ, Mimnaugh EG, Martinez A, Cuttitta F, Neckers LM (2002). Hsp90 regulates a von Hippel Lindau-independent hypoxia-inducible factor-1 alpha-degradative pathway. J Biol Chem. 277(33): 29936-44.

Ivan M, Haberberger T, Gervasi DC, Michelson KS, Gunzler V, Kondo K, Yang H, Sorokina I, Conaway RC, Conaway JW, Kaelin WG, Jr. (2002). Biochemical purification and pharmacological inhibition of a mammalian prolyl hydroxylase acting on hypoxia-inducible factor. Proc Natl Acad Sci U S A. 99(21): 13459-64.

Ivan M, Kondo K, Yang H, Kim W, Valiando J, Ohh M, Salic A, Asara JM, Lane WS, Kaelin WG, Jr. (2001). HIFα targeted for VHL-mediated destruction by proline hydroxylation: implications for O2 sensing. Science 292(5516): 464-8.

Iyer NV, Kotch LE, Agani F, Leung SW, Laughner E, Wenger RH, Gassmann M, Gearhart JD, Lawler AM, Yu AY, Semenza GL (1998). Cellular and developmental control of O2 homeostasis by hypoxia-inducible factor 1 alpha. Genes Dev. 12(2): 149-62.

Jaakkola P, Mole DR, Tian YM, Wilson MI, Gielbert J, Gaskell SJ, Kriegsheim A, Hebestreit HF, Mukherji M, Schofield CJ, Maxwell PH, Pugh CW, Ratcliffe PJ (2001). Targeting of HIF-α to the von Hippel-Lindau ubiquitylation complex by O2-regulated prolyl hydroxylation. Science 292(5516): 468-72.

Jelkmann W (2007). Erythropoietin after a century of research: younger than ever. Eur J Haematol. 78(3): 183-205.

Jiang BH, Semenza GL, Bauer C, Marti HH (1996). Hypoxia-inducible factor 1 levels vary exponentially over a physiologically relevant range of O2 tension. Am J Physiol. 271(4 Pt 1): C1172-80.

Kaelin WG, Jr., Ratcliffe PJ (2008). Oxygen sensing by metazoans: the central role of the HIF hydroxylase pathway. Mol Cell. 30(4): 393-402.

Kimura H, Weisz A, Ogura T, Hitomi Y, Kurashima Y, Hashimoto K, D'Acquisto F, Makuuchi M, Esumi H (2001). Identification of hypoxia-inducible factor 1 ancillary sequence and its function in vascular endothelial growth factor gene induction by hypoxia and nitric oxide. J Biol Chem. 276(3): 2292-8.

Knowles HJ, Raval RR, Harris AL, Ratcliffe PJ (2003). Effect of ascorbate on the activity of hypoxia-inducible factor in cancer cells. Cancer Res 63(8): 1764-8.

Koditz J, Nesper J, Wottawa M, Stiehl DP, Camenisch G, Franke C, Myllyharju J, Wenger RH, Katschinski DM (2007). Oxygen-dependent ATF-4 stability is mediated by the PHD3 oxygen sensor. Blood. 110(10): 3610-7.

Koivunen P, Hirsila M, Remes AM, Hassinen IE, Kivirikko KI, Myllyharju J (2007). Inhibition of hypoxia-inducible factor (HIF) hydroxylases by citric acid cycle intermediates: possible links between cell metabolism and stabilization of HIF. J Biol Chem. 282(7): 4524-32.

Koivunen P, Tiainen P, Hyvarinen J, Williams KE, Sormunen R, Klaus SJ, Kivirikko KI, Myllyharju J (2007). An endoplasmic reticulum transmembrane prolyl 4-hydroxylase is induced by hypoxia and acts on hypoxia-inducible factor alpha. J Biol Chem. 282(42): 30544-52.

Kotch LE, Iyer NV, Laughner E, Semenza GL (1999). Defective vascularization of HIF-1alpha-null embryos is not associated with VEGF deficiency but with mesenchymal cell death. Dev Biol. 209(2): 254-67.

Kozak KR, Abbott B, Hankinson O (1997). ARNT-deficient mice and placental differentiation. Dev Biol. 191(2): 297-305.

Kuiper C, Molenaar IG, Dachs GU, Currie MJ, Sykes PH, Vissers MC (2010). Low Ascorbate Levels Are Associated with Increased Hypoxia-Inducible Factor-1 Activity and an Aggressive Tumor Phenotype in Endometrial Cancer. Cancer Res 2010: 22.

Ladroue C, Carcenac R, Leporrier M, Gad S, Le Hello C, Galateau-Salle F, Feunteun J, Pouysségur J, Richard S, Gardie B (2008). PHD2 mutation and congenital erythrocytosis with paraganglioma. N Engl J Med 359(25): 2685-92.

Lando D, Peet DJ, Gorman JJ, Whelan DA, Whitelaw ML, Bruick RK (2002). FIH-1 is an asparaginyl hydroxylase enzyme that regulates the transcriptional activity of hypoxia-inducible factor. Genes Dev. 16(12): 1466-71.

Lee PJ, Jiang BH, Chin BY, Iyer NV, Alam J, Semenza GL, Choi AM (1997). Hypoxia-inducible factor-1 mediates transcriptional activation of the heme oxygenase-1 gene in response to hypoxia. J Biol Chem. 272(9): 5375-81.

Linster CL, Van Schaftingen E (2007). Vitamin C. Biosynthesis, recycling and degradation in mammals. Febs J. 274(1): 1-22.

Lisy K, Peet DJ, Bracken CP, Fedele AO, Linke S, Balrak W, Lisy K, Whitelaw ML, Peet DJ, Blaskovic D, Sekeyova Z, Kozuch O, Labuda M, Lisy K, Cupalova A, Mazak V, Vlcek M, Chmela J, Hubalek Z, et al. (2008). Turn me on: regulating HIF transcriptional activity. Cell Death Differ. 15(4):642-9.

Mahon PC, Hirota K, Semenza GL (2001). FIH-1: a novel protein that interacts with HIF-1alpha and VHL to mediate repression of HIF-1 transcriptional activity. Genes Dev. 15(20): 2675-86.

Makino Y, Cao R, Svensson K, Bertilsson G, Asman M, Tanaka H, Cao Y, Berkenstam A, Poellinger L (2001). Inhibitory PAS domain protein is a negative regulator of hypoxia-inducible gene expression. Nature. 414(6863): 550-4.

Martin F, Linden T, Katschinski DM, Oehme F, Flamme I, Mukhopadhyay CK, Eckhardt K, Troger J, Barth S, Camenisch G, Wenger RH (2005). Copper-dependent activation of hypoxia-inducible factor (HIF)-1: implications for ceruloplasmin regulation. Blood. 105(12): 4613-9.

Masson N, Appelhoff RJ, Tuckerman JR, Tian YM, Demol H, Puype M, Vandekerckhove J, Ratcliffe PJ, Pugh CW (2004). The HIF prolyl hydroxylase PHD3 is a potential substrate of the TRiC chaperonin. FEBS Lett. 570(1-3): 166-70.

Masson N, Willam C, Maxwell PH, Pugh CW, Ratcliffe PJ (2001). Independent function of two destruction domains in hypoxia-inducible factor-alpha chains activated by prolyl hydroxylation. Embo J. 20(18): 5197-206.

Maxwell PH, Pugh CW, Ratcliffe PJ (1993). Inducible operation of the erythropoietin 3' enhancer in multiple cell lines: evidence for a widespread oxygen-sensing mechanism. Proc Natl Acad Sci U S A. 90(6): 2423-7.

Mazzone M, Dettori D, Leite de Oliveira R, Loges S, Schmidt T, Jonckx B, Tian YM, Lanahan AA, Pollard P, Ruiz de Almodovar C, De Smet F, Vinckier S, Aragones J, Debackere K, Luttun A, Wyns S, Jordan B, Pisacane A, Gallez B, Lampugnani MG, Dejana E, Simons M, Ratcliffe P, Maxwell P, Carmeliet P (2009). Heterozygous deficiency of PHD2 restores tumor oxygenation and inhibits metastasis via endothelial normalization. Cell. 136(5): 839-51.

McDonough MA, Li V, Flashman E, Chowdhury R, Mohr C, Lienard BM, Zondlo J, Oldham NJ, Clifton IJ, Lewis J, McNeill LA, Kurzeja RJ, Hewitson KS, Yang E, Jordan S, Syed RS, Schofield CJ (2006). Cellular oxygen sensing: Crystal structure of hypoxia-inducible factor prolyl hydroxylase (PHD2). Proc Natl Acad Sci U S A. 103(26): 9814-9.

McMahon S, Charbonneau M, Grandmont S, Richard DE, Dubois CM (2006). Transforming growth factor beta1 induces hypoxia-inducible factor-1 stabilization through selective inhibition of PHD2 expression. J Biol Chem. 281(34): 24171-81.

McNeill LA, Flashman E, Buck MR, Hewitson KS, Clifton IJ, Jeschke G, Claridge TD, Ehrismann D, Oldham NJ, Schofield CJ (2005). Hypoxia-inducible factor prolyl hydroxylase 2 has a high affinity for ferrous iron and 2-oxoglutarate. Mol Biosyst. 1(4): 321-4.

Melillo G, Taylor LS, Brooks A, Musso T, Cox GW, Varesio L (1997). Functional requirement of the hypoxia-responsive element in the activation of the inducible nitric oxide synthase promoter by the iron chelator desferrioxamine. J Biol Chem. 272(18): 12236-43.

Metzen E (2007). Enzyme substrate recognition in oxygen sensing: how the HIF trap snaps. Biochem J. 408(2): e5-6.

Metzen E, Berchner-Pfannschmidt U, Stengel P, Marxsen JH, Stolze I, Klinger M, Huang WQ, Wotzlaw C, Hellwig-Burgel T, Jelkmann W, Acker H, Fandrey J (2003). Intracellular localisation of human HIF-1 alpha hydroxylases: implications for oxygen sensing. J Cell Sci. 116(Pt 7): 1319-26.

Metzen E, Stiehl DP, Doege K, Marxsen JH, Hellwig-Burgel T, Jelkmann W (2005). Regulation of the prolyl hydroxylase domain protein 2 (phd2/egln-1) gene:

identification of a functional hypoxia-responsive element. Biochem J. 387(Pt 3): 711-7.

Mikhaylova O, Ignacak ML, Barankiewicz TJ, Harbaugh SV, Yi Y, Maxwell PH, Schneider M, Van Geyte K, Carmeliet P, Revelo MP, Wyder M, Greis KD, Meller J, Czyzyk-Krzeska MF (2008). The von Hippel-Lindau tumor suppressor protein and Egl-9-Type proline hydroxylases regulate the large subunit of RNA polymerase II in response to oxidative stress. Mol Cell Biol. 28(8): 2701-17.

Minamishima YA, Moslehi J, Padera RF, Bronson RT, Liao R, Kaelin WG, Jr. (2009). A feedback loop involving the Phd3 prolyl hydroxylase tunes the mammalian hypoxic response in vivo. Mol Cell Biol. 29(21): 5729-41.

Myllyharju J (2008). Prolyl 4-hydroxylases, key enzymes in the synthesis of collagens and regulation of the response to hypoxia, and their roles as treatment targets. Ann Med 23: 1-16.

Myllylä R, Majamaa K, Günzler V, Hanauske-Abel HM, Kivirikko KI (1984). Ascorbate is consumed stoichiometrically in the uncoupled reactions catalyzed by prolyl 4-hydroxylase and lysyl hydroxylase. J Biol Chem 259(9): 5403-5.

Mylonis I, Chachami G, Samiotaki M, Panayotou G, Paraskeva E, Kalousi A, Georgatsou E, Bonanou S, Simos G (2006). Identification of MAPK phosphorylation sites and their role in the localization and activity of hypoxia-inducible factor-1alpha. J Biol Chem. 281(44): 33095-106.

Nagel S, Talbot NP, Mecinovic J, Smith TG, Buchan AM, Schofield CJ (2010). Therapeutic manipulation of the HIF hydroxylases. Antioxid 12(4): 481-501.

Nakayama K, Frew IJ, Hagensen M, Skals M, Habelhah H, Bhoumik A, Kadoya T, Erdjument-Bromage H, Tempst P, Frappell PB, Bowtell DD, Ronai Z (2004). Siah2 regulates stability of prolyl-hydroxylases, controls HIF1alpha abundance, and modulates physiological responses to hypoxia. Cell. 117(7): 941-52.

Nytko KJ, Spielmann P, Camenisch G, Wenger RH, Stiehl DP (2007). Regulated function of the prolyl-4-hydroxylase domain (PHD) oxygen sensor proteins. Antioxid Redox Signal 9(9): 1329-38.

Oehme F, Ellinghaus P, Kolkhof P, Smith TJ, Ramakrishnan S, Hutter J, Schramm M, Flamme I (2002). Overexpression of PH-4, a novel putative proline 4-hydroxylase, modulates activity of hypoxia-inducible transcription factors. Biochem Biophys Res Commun. 296(2): 343-9.

Oehme F, Jonghaus W, Narouz-Ott L, Huetter J, Flamme I (2004). A nonradioactive 96-well plate assay for the detection of hypoxia-inducible factor prolyl hydroxylase activity. Anal Biochem. 330(1): 74-80.

Ozer A, Wu LC, Bruick RK (2005). The candidate tumor suppressor ING4 represses activation of the hypoxia inducible factor (HIF). Proc Natl Acad Sci U S A. 102(21): 7481-6.

Pan Y, Mansfield KD, Bertozzi CC, Rudenko V, Chan DA, Giaccia AJ, Simon MC (2007). Multiple factors affecting cellular redox status and energy metabolism modulate hypoxia-inducible factor prolyl hydroxylase activity in vivo and in vitro. Mol Cell Biol 27(3): 912-25.

Percy MJ, Furlow PW, Beer PA, Lappin TR, McMullin MF, Lee FS (2007). A novel erythrocytosis-associated PHD2 mutation suggests the location of a HIF binding groove. Blood 110(6): 2193-6.

Percy MJ, Zhao Q, Flores A, Harrison C, Lappin TR, Maxwell PH, McMullin MF, Lee FS (2006). A family with erythrocytosis establishes a role for prolyl

hydroxylase domain protein 2 in oxygen homeostasis. Proc Natl Acad Sci USA 103(3): 654-9.

Pescador N, Cuevas Y, Naranjo S, Alcaide M, Villar D, Landazuri MO, Del Peso L (2005). Identification of a functional hypoxia-responsive element that regulates the expression of the egl nine homologue 3 (egln3/phd3) gene. Biochem J. 390(Pt 1): 189-97.

Pollard PJ, Briere JJ, Alam NA, Barwell J, Barclay E, Wortham NC, Hunt T, Mitchell M, Olpin S, Moat SJ, Hargreaves IP, Heales SJ, Chung YL, Griffiths JR, Dalgleish A, McGrath JA, Gleeson MJ, Hodgson SV, Poulsom R, Rustin P, Tomlinson IP (2005). Accumulation of Krebs cycle intermediates and over-expression of HIF1alpha in tumours which result from germline FH and SDH mutations. Hum Mol Genet. 14(15): 2231-9.

Pollard PJ, Spencer-Dene B, Shukla D, Howarth K, Nye E, El-Bahrawy M, Deheragoda M, Joannou M, McDonald S, Martin A, Igarashi P, Varsani-Brown S, Rosewell I, Poulsom R, Maxwell P, Stamp GW, Tomlinson IP (2007). Targeted inactivation of fh1 causes proliferative renal cyst development and activation of the hypoxia pathway. Cancer Cell. 11(4): 311-9.

Priestley J (1775). An Account of Further Discoveries in Air. Philosophical Transactions (1683-1775); 65: 384-394.

Pugh CW, O'Rourke JF, Nagao M, Gleadle JM, Ratcliffe PJ (1997). Activation of hypoxia-inducible factor-1; definition of regulatory domains within the alpha subunit. J Biol Chem. 272(17): 11205-14.

Richard DE, Berra E, Gothie E, Roux D, Pouyssegur J (1999). p42/p44 mitogen-activated protein kinases phosphorylate hypoxia-inducible factor 1alpha (HIF-1alpha) and enhance the transcriptional activity of HIF-1. J Biol Chem. 274(46): 32631-7.

Rolfs A, Kvietikova I, Gassmann M, Wenger RH (1997). Oxygen-regulated transferrin expression is mediated by hypoxia-inducible factor-1. J Biol Chem. 272(32): 20055-62.

Salnikow K, Donald SP, Bruick RK, Zhitkovich A, Phang JM, Kasprzak KS (2004). Depletion of intracellular ascorbate by the carcinogenic metals nickel and cobalt results in the induction of hypoxic stress. J Biol Chem 279(39): 40337-44.

Sang N, Stiehl DP, Bohensky J, Leshchinsky I, Srinivas V, Caro J (2003). MAPK signaling up-regulates the activity of hypoxia-inducible factors by its effects on p300. J Biol Chem. 278(16): 14013-9.

Scheele CW (1777). Chemical Treatise of Air and Fire (Ger), Upsala and Leipzig, M Swederus.

Schlisio S, Kenchappa RS, Vredeveld LC, George RE, Stewart R, Greulich H, et al. The kinesin KIF1Bβ acts downstream from EglN3 to induce apoptosis and is a potential 1p36 tumor suppressor. Genes Dev 2008 22(7): 884-93.

Schneider M, Van Geyte K, Fraisl P, Kiss J, Aragones J, Mazzone M, Mairbaurl H, De Bock K, Jeoung NH, Mollenhauer M, Georgiadou M, Bishop T, Roncal C, Sutherland A, Jordan B, Gallez B, Weitz J, Harris RA, Maxwell P, Baes M, Ratcliffe P, Carmeliet P (1143). Loss or silencing of the PHD1 prolyl hydroxylase protects livers of mice against ischemia/reperfusion injury. Gastroenterology 138(3): 1143-54.

Scortegagna M, Ding K, Oktay Y, Gaur A, Thurmond F, Yan LJ, Marck BT, Matsumoto AM, Shelton JM, Richardson JA, Bennett MJ, Garcia JA (2003).

Multiple organ pathology, metabolic abnormalities and impaired homeostasis of reactive oxygen species in Epas1-/- mice. Nat Genet. 35(4): 331-40.

Semenza GL, Nejfelt MK, Chi SM, Antonarakis SE (1991). Hypoxia-inducible nuclear factors bind to an enhancer element located 3' to the human erythropoietin gene. Proc Natl Acad Sci USA 88(13): 5680-4.

Semenza GL, Roth PH, Fang HM, Wang GL (1994). Transcriptional regulation of genes encoding glycolytic enzymes by hypoxia-inducible factor 1. J Biol Chem. 269(38): 23757-63.

Semenza GL, Wang GL (1992). A nuclear factor induced by hypoxia via de novo protein synthesis binds to the human erythropoietin gene enhancer at a site required for transcriptional activation. Mol Cell Biol. 12(12): 5447-54.

Soilleux EJ, Turley H, Tian YM, Pugh CW, Gatter KC, Harris AL (2005). Use of novel monoclonal antibodies to determine the expression and distribution of the hypoxia regulatory factors PHD-1, PHD-2, PHD-3 and FIH in normal and neoplastic human tissues. Histopathology. 47(6): 602-10.

Srinivas V, Leshchinsky I, Sang N, King MP, Minchenko A, Caro J (2001). Oxygen sensing and HIF-1 activation does not require an active mitochondrial respiratory chain electron-transfer pathway. J Biol Chem. 276(25): 21995-8.

Stiehl DP, Jelkmann W, Wenger RH, Hellwig-Burgel T (2002). Normoxic induction of the hypoxia-inducible factor 1alpha by insulin and interleukin-1beta involves the phosphatidylinositol 3-kinase pathway. FEBS Lett 512(1-3): 157-62.

Stiehl DP, Wirthner R, Köditz J, Spielmann P, Camenisch G, Wenger RH (2006). Increased prolyl 4-hydroxylase domain proteins compensate for decreased oxygen levels. Evidence for an autoregulatory oxygen-sensing system. J Biol Chem 281(33): 23482-91.

Stroka DM, Burkhardt T, Desbaillets I, Wenger RH, Neil DA, Bauer C, Gassmann M, Candinas D (2001). HIF-1 is expressed in normoxic tissue and displays an organ-specific regulation under systemic hypoxia. Faseb J. 15(13): 2445-53.

Takeda K, Aguila HL, Parikh NS, Li X, Lamothe K, Duan LJ, Takeda H, Lee FS, Fong GH (2008). Regulation of adult erythropoiesis by prolyl hydroxylase domain proteins. Blood 111(6): 3229-35.

Takeda K, Cowan A, Fong GH (2007). Essential role for prolyl hydroxylase domain protein 2 in oxygen homeostasis of the adult vascular system. Circulation 116(7): 774-81.

Takeda K, Ho VC, Takeda H, Duan LJ, Nagy A, Fong GH (2006). Placental but not heart defects are associated with elevated hypoxia-inducible factor α levels in mice lacking prolyl hydroxylase domain protein 2. Mol Cell Biol 26(22): 8336-46.

Tian H, Hammer RE, Matsumoto AM, Russell DW, McKnight SL (1998). The hypoxia-responsive transcription factor EPAS1 is essential for catecholamine homeostasis and protection against heart failure during embryonic development. Genes Dev. 12(21): 3320-4.

Tian H, McKnight SL, Russell DW (1997). Endothelial PAS domain protein 1 (EPAS1), a transcription factor selectively expressed in endothelial cells. Genes Dev. 11(1): 72-82.

Tsukiyama F, Nakai Y, Yoshida M, Tokuhara T, Hirota K, Sakai A, Hayashi H, Katsumata T (2006). Gallate, the component of HIF-inducing catechins, inhibits HIF prolyl hydroxylase. Biochem Biophys Res Commun. 351(1): 234-9.

Vaux EC, Metzen E, Yeates KM, Ratcliffe PJ (2001). Regulation of hypoxia-inducible factor is preserved in the absence of a functioning mitochondrial respiratory chain. Blood. 98(2): 296-302.

Villar D, Vara-Vega A, Landazuri MO, Del Peso L (2007). Identification of a region on hypoxia-inducible-factor prolyl 4-hydroxylases that determines their specificity for the oxygen degradation domains. Biochem J. 408(2): 231-40.

Wang GL, Jiang BH, Rue EA, Semenza GL (1995). Hypoxia-inducible factor 1 is a basic-helix-loop-helix-PAS heterodimer regulated by cellular O2 tension. Proc Natl Acad Sci U S A. 92(12): 5510-4.

Wang GL, Semenza GL (1995). Purification and characterization of hypoxia-inducible factor 1. J Biol Chem. 270(3): 1230-7.

Wenger RH, Camenisch G, Stiehl DP, Katschinski DM (2009). HIF prolyl-4-hydroxylase interacting proteins: consequences for drug targeting. Curr Pharm Des. 15(33): 3886-94.

Wenger RH, Stiehl DP, Camenisch G (2005). Integration of oxygen signaling at the consensus HRE. Sci STKE 2005(306): re12.

Wiesener MS, Jurgensen JS, Rosenberger C, Scholze CK, Horstrup JH, Warnecke C, Mandriota S, Bechmann I, Frei UA, Pugh CW, Ratcliffe PJ, Bachmann S, Maxwell PH, Eckardt KU (2003). Widespread hypoxia-inducible expression of HIF-2alpha in distinct cell populations of different organs. Faseb J. 17(2): 271-3.

Wingrove JA, O'Farrell PH (1999). Nitric oxide contributes to behavioral, cellular, and developmental responses to low oxygen in Drosophila. Cell. 98(1): 105-14.

Yasinska IM, Sumbayev VV (2003). S-nitrosation of Cys-800 of HIF-1alpha protein activates its interaction with p300 and stimulates its transcriptional activity. FEBS Lett. 549(1-3): 105-9.

Yu AY, Frid MG, Shimoda LA, Wiener CM, Stenmark K, Semenza GL (1998). Temporal, spatial, and oxygen-regulated expression of hypoxia-inducible factor-1 in the lung. Am J Physiol. 275(4 Pt 1): L818-26.

Yu F, White SB, Zhao Q, Lee FS (2001). HIF-1alpha binding to VHL is regulated by stimulus-sensitive proline hydroxylation. Proc Natl Acad Sci U S A. 98(17): 9630-5.

Yuan Y, Hilliard G, Ferguson T, Millhorn DE (2003). Cobalt inhibits the interaction between hypoxia-inducible factor-alpha and von Hippel-Lindau protein by direct binding to hypoxia-inducible factor-alpha. J Biol Chem. 278(18): 15911-6.

5. Aims of the study

I. Characterization of hydroxylation activity of recombinant PHD isoforms in the context of requirements for essential co-substrates and co-factors using an *in vitro* hydroxylation assay.

II. Studies on the influence of small molecule antioxidants, reactive oxygen species, transition metals and metal chelators on PHDs hydroxylation activity *in vitro.*

III. Analysis of the role of ascorbate and other antioxidants in regulating cellular oxygen sensing via the HIF/PHDs pathway in living cells and *in vivo.*

6. Manuscript I: Regulated Function of the Prolyl-4-hydroxylase Domain (PHD) Oxygen Sensor Proteins

Katarzyna J. Nytko, Patrick Spielmann, Gieri Camenisch, Roland H. Wenger , and Daniel P. Stiehl

Department of Physiology and Zürich Center for Integrative Human Physiology ZIHP, University of Zürich, CH-8057 Zürich, Switzerland.

Running title:

REGULATED FUNCTION OF THE PHD OXYGEN SENSORS

Correspondence:
Roland H. Wenger,
Institute of Physiology,
University of Zürich-Irchel,
Winterthurerstrasse 190,
CH-8057 Zürich, Switzerland.
Tel: +41 (0)44 63 55065;
Fax: +41 (0)44 63 56814;
E-Mail: roland.wenger@access.unizh.ch

Abstract

Cellular oxygen is sensed by prolyl-4-hydroxylase domain (PHD) proteins that hydroxylate hypoxia-inducible factor (HIF) α subunits. Under normoxic conditions, hydroxylated HIFα is bound by the von Hippel-Lindau (pVHL) tumor suppressor, leading to ubiquitinylation and proteasomal degradation. Under hypoxic conditions hydroxylation becomes reduced, leading to HIFα stabilization. We recently showed that changes in PHD abundance and activity can regulate HIFα stability under normoxic as well as under hypoxic conditions. Thus, the PHD oxygen sensors themselves represent effectors of cellular signalling pathways as well as potential drug targets. Here, we applied a cell-free *in vitro* microtiter plate-based peptide hydroxylation assay to investigate the influence of ferrous iron, Krebs cycle intermediates, transition metals, vitamin C and other antioxidants on the activity of purified PHD1 to 3. PHD activity depends not only on oxygen availability but is also regulated by iron, vitamin C and Krebs cycle intermediates, suggesting a physiological relevance of their cellular concentrations. Copper but not iron, cobalt or nickel salts catalyzed vitamin C oxidation. While vitamin C is essential for PHD activity *in vitro*, N-acetyl-L-cysteine had no effect, and gallic acid or n-propyl gallate efficiently inhibited the activity of all three PHDs, demonstrating different functions of these antioxidants.

Introduction

Oxygen availability affects many physiological and pathophysiological processes, including embryonic development, adaptation to high-altitudes, wound healing, inflammation, cancer and ischemic diseases such as infarction and stroke. Central to the understanding of these processes is the elucidation of the molecular mechanisms by which cells react and adapt to insufficient oxygen supply (hypoxia). Oxygen availability is measured by a family of oxygen-dependent protein hydroxylases that regulate the abundance and activity of hypoxia-inducible transcription factor (HIF) α subunits (Schofield and Ratcliffe, 2004). HIFs in turn control the expression levels of effector genes involved in either anticipatory metabolic changes, adaptive survival or programmed death of the affected tissue (Wenger et al., 2005). HIFs are heterodimeric transcription factors consisting of one out of three different oxygen-sensitive HIFα subunits (HIF-1α, HIF-2α or HIF-3α) and a common constitutive HIFβ subunit. While HIF-1 and HIF-2 αβ heterodimers function as transcriptional activators of oxygen-regulated target genes, the role of HIF-3α is less clear and a short splice variant of HIF-3α, termed inhibitory PAS protein (IPAS), functions as a transcriptional repressor (Wenger, 2002).

Dependent upon the cellular oxygen partial pressure (pO_2), a family of prolyl-4-hydroxylase domain (PHD) enzymes covalently modify two proline residues within the oxygen-dependent degradation (ODD) domain of HIFα subunits. The PHD family is comprised of three members called PHD1, PHD2, PHD3, or HIF prolyl hydroxylase (HPH) HPH3, HPH2, HPH1, respectively (Bruick and McKnight, 2001; Epstein et al., 2001). A fourth member, called PH-4, regulates HIFα under overexpression conditions only (Oehme et al., 2002). Upon hydroxylation under normoxic conditions, HIFα is bound by the von Hippel-Lindau (VHL) tumor suppressor protein and targeted for proteasomal destruction (Maxwell et al., 1999). Thus, the high turnover rate of HIFα subunits allows for an instantaneous stabilization under hypoxic conditions (Jewell et al., 2001). According to the current model, also the asparagine hydroxylase function of the factor inhibiting HIF (FIH) becomes impaired when oxygen availability is further decreased, resulting in a decrease in C-terminal HIFα asparagine hydroxylation (Pouyssegur et al., 2006). This allows for the progressively increased recruitment of p300/CPB transcriptional co-activators,

leading to a successively higher transcriptional function of HIF (Lando et al., 2002; Mahon et al., 2001).

While all three PHDs can hydroxylate HIFα with similar efficiency, PHD2 has been suggested to play the main role for normoxic HIFα turnover (Berra et al., 2003). Consistent with these *in vitro* findings, PHD2 but not PHD1 or PHD3 knock-out mice die during embryonic development (Takeda et al., 2006). The three PHDs are expressed in most organs but there are strikingly high levels of PHD3 mRNA in the heart and of PHD1 mRNA in the testis (Stiehl et al., 2006). In addition to HIFα, there is some evidence that the iron regulatory protein IRP2, the RNApol II large subunit Rpb1, the heme synthesis enzyme ALAS2, and IκB kinase-β are regulated by PHDs (Abu-Farha et al., 2005; Cummins et al., 2006; Kuznetsova et al., 2003; Wang et al., 2004; Wang and Pantopoulos, 2005). Of note, these experiments were mainly based on pharmacological inhibition of PHDs and no direct evidence for protein hydroxylation has been provided yet. Interestingly, ankyrin repeats within the NF-κB family (p105) and IκBα were shown to be efficiently hydroxylated by FIH (Cockman et al., 2000). The function of this FIH-dependent hydroxylation, however, is unclear up to date.

The regulation of PHD expression and activity has become of considerable interest in the recent past. Endogenous tricarboxylic acid cycle intermediates and reactive oxygen species (ROS) have been reported to inhibit PHD function and hence link mitochondrial function with PHD-dependent oxygen sensing (Dalgard et al., 2004; Gerald et al., 2004; Pollard et al., 2005; Selak et al., 2005). Small molecule inhibitors that can be added exogenously are currently being developed for clinical tissue protection in diseases associated with oxygen deprivation. However, little is known on the differential regulation of the three PHD family members. We therefore set out to investigate the control of each PHD family member individually by small molecules as well as by newly identified protein interactors and by HIF-dependent feedback regulation.

Materials and methods

Expression and purification of PHDs

GST-PHD1, GST-PHD2 and GST-PHD3 were expressed in baculovirus-infected Sf9 insect cells and purified as described before (Stiehl et al., 2006). Briefly, after 80 to 110 hours of infection Sf9 cells were lysed in ice-cold 0.1% NP-40, 10 mM Tris-HCl pH 7.5, 100 mM NaCl, 100 mM glycine and 10 μM DTT. Cleared lysates were incubated with equilibrated glutathione-sepharose beads (Amersham, Dübendorf, Switzerland) for 2 hours at 4°C with gentle agitation. After washing of the beads three times with PBS, bound protein was eluted with 15 mM reduced glutathione, 50 mM Tris-HCl pH 8.0, and 2 μM $FeSO_4$. Purity of recombinant fusion-proteins was routinely estimated by SDS-PAGE and coomassie blue staining.

In vitro prolyl-4-hydroxylation assays

Enzymatic activity of recombinant PHDs was determined as described before (Martin et al., 2005; Oehme et al., 2004). Biotinylated peptides (100 ng/well) derived from human HIF-1α amino acids 556 to 574 (either wild-type or P564A mutant) were bound to NeutrAvidin-coated 96-well plates (Pierce, Perbio, Lausanne, Switzerland). Purified recombinant PHD enzymes were used to hydroxylate the peptides in the presence of 0.5 mM 2-oxoglutarate, 2 mM ascorbate, 10 μM $FeSO_4$ in 20 mM Tris-HCl pH 7.5, 5 mM KCl, 1.5 mM $MgCl_2$ for 1 hour at room temperature. A polycistronic expression vector for His_6- and thioredoxin-tagged pVHL/elongin B/elongin C (VBC) complex was kindly provided by S. Tan (Pennsylvania, PA). VBC was expressed in bacteria, purified by nickel affinity chromatography followed by ion exchange chromatography and buffer exchange gelfiltration. VBC complex was allowed to bind to the hydroxylated peptides and bound VBC complex was detected by rabbit anti-thioredoxin antibodies followed by secondary horseradish peroxidase-coupled anti-rabbit antibodies (Sigma, Buchs, Switzerland), using the TMB (3,3',5,5'-tetramethylbenzidine) substrate kit (Pierce). The peroxidase reaction was stopped by adding H_2SO_4 to 1 M and absorbance was determined at 450 nm in a microplate reader.

Ascorbate oxidation assays

Ascorbate oxidation to dehydroascorbate was measured spectrophotometrically as described (Stait and Leake, 1996). Therefore, the decrease in absorbance at 265 nm was measured in a 100 μM ascorbate solution in 20 mM Tris-HCl pH 7.5, 5 mM KCl, 1.5 mM $MgCl_2$ using open cuvettes with free access to air.

Hypoxia reporter cells

The chinese hamster ovary (CHO) cell line stably transfected with a hypoxia-responsive firefly luciferase reporter gene (termed HRCHO5) has been described before (Wanner et al., 2000). Cell lysis and determination of luciferase activity was performed according to the manufacturer's instructions (Promega, Wallisellen, Switzerland). Relative light units were measured in a 96-well luminometer (Berthold, Regensdorf, Switzerland) and normalized to the protein concentration determined by the Bradford assay (Biorad, Reinach, Switzerland).

Results

Ferrous iron availability is essential for the function of all three PHDs

GST-tagged PHD1, PHD2 and PHD3 were purified from Sf9 insect cells to 80-90% purity and their activity was analyzed by a VBC binding assay. All three PHDs induced VBC binding to the wild-type P564 but not to the mutant P564A-containing peptide derived from the HIF-1α ODD (Fig. 1a). The PHD preparations were calibrated with hydroxyproline (Hyp)564 peptide-containing hydroxylation assays and subsequently diluted to obtain solutions with similar specific activities which were then used for all following experiments.

Although 2 μM $FeSO_4$ was initially present in the elution buffer, the PHDs did not display full activity without additional ferrous iron in the reaction buffer (Fig. 1b), suggesting that both active center and added iron was at least partially oxidized. An iron titration experiment revealed that the addition of ~ 10 μM $FeSO_4$ was required for full induction of the hydroxylation activity of all three PHDs (Fig. 1c). This concentration was hence kept in the following experiments. The addition of ferric iron did not stimulate the activity of the PHDs (data not shown).

The sensitivity of the PHDs to the availability of "free" iron explains the long-known feature of iron chelators to induce the HIFα subunits. Indeed, the hexadentate hydroxamic acid iron chelator desferrioxamine inhibited the *in vitro* hydroxylation activity of all three PHDs (Fig. 1d). Of note, PHD2 was somewhat less sensitive to desferrioxamine than PHD1 or PHD3 which were efficiently inhibited by 10 μM desferrioxamine, corresponding to the concentration of iron in the reaction solution.

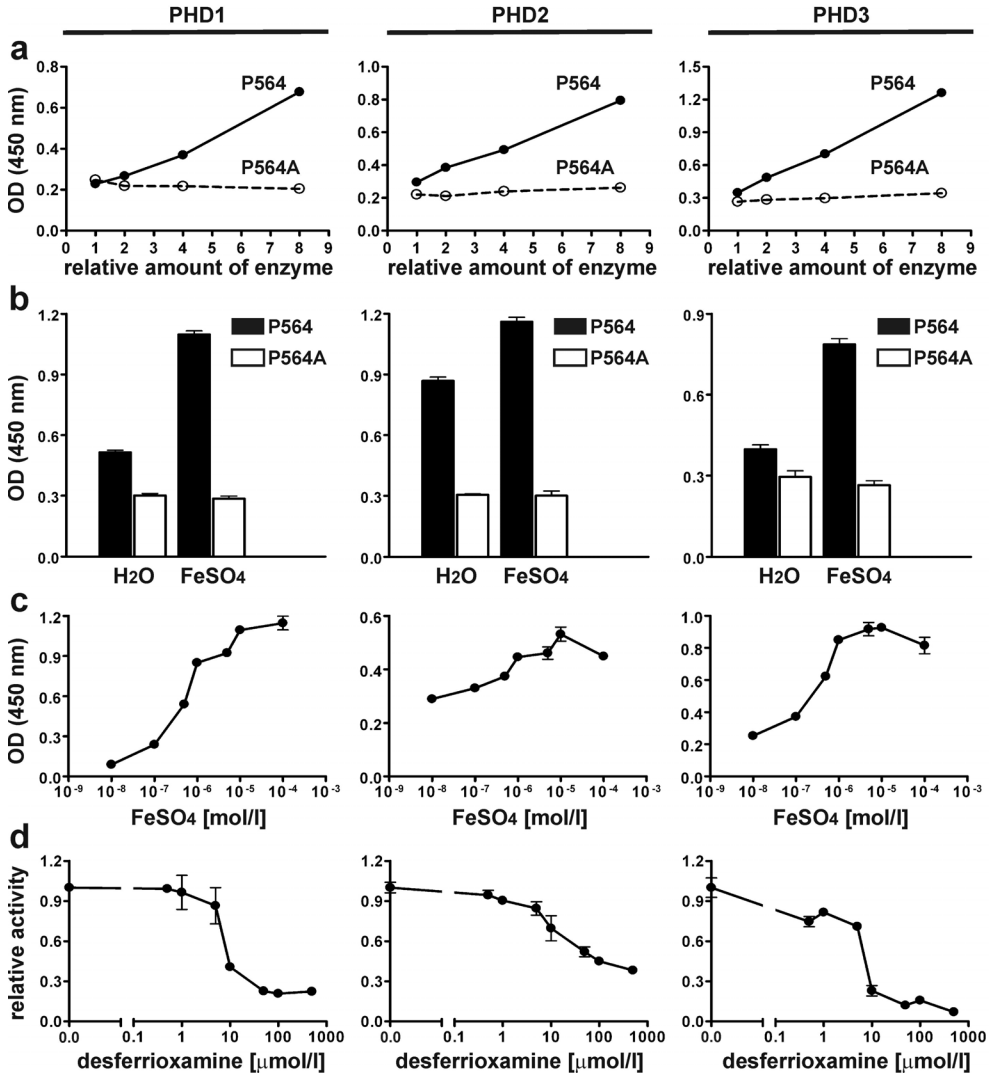

Fig. 1 Regulation of PHD function by iron. PHD1, PHD2 and PHD3 (from left to right) were purified as GST-tagged fusion-proteins from Sf9 cells and their hydroxylation activity was estimated in microtiter plate-based VBC binding assays. **(a)** VBC binding is dependent on the presence of both functional PHD enzyme and Pro564. Mutant P564A peptides cannot be hydroxylated. **(b)** Ferrous iron supplementation is essential for full PHD function. **(c)** Titration of ferrous iron reveals that ~ 10 μM $FeSO_4$ is required for full PHD function. **(d)** Inhibition of PHD function by iron chelation. Mean values ± SEM of representative experiments performed in triplicates are shown.

Requirement for 2-oxoglutarate and inhibition by succinate

2-Oxoglutarate is used as a co-substrate of all three PHDs which is oxidatively decarboxylated during target protein hydroxylation. Titration experiments with 2-oxoglutarate revealed that all three PHDs required a similar 2-oxoglutarate concentration of ~ 10-100 μM for full activity (Fig. 2a). These data confirm previous estimates of Km values of 55-60 μM for all three PHDs (Hirsilä et al., 2003), and a PHD2 binding constant for 2-oxoglutarate of < 2 μM (McNeill et al., 2005). Therefore, a concentration of 500 μM 2-oxoglutarate was kept in all further experiments. Higher concentrations of 2-oxoglutarate inhibited the *in vitro* PHD activity in some experiments. Whether this effect is of physiological relevance is currently unknown.

It has been reported previously that succinate can inhibit PHD activity (Dalgard et al., 2004; Lu et al., 2005; Selak et al., 2005). We hence tested the effects of increasing succinate concentration on each PHD enzyme. In the presence of 500 μM 2-oxoglutarate, addition of succinate only moderately inhibited the hydroxylation activity of purified PHDs and this effect was even reversed at higher concentrations (Fig. 2b). However, in the presence of 50 μM 2-oxoglutarate, succinate efficiently inhibited PHD activity with IC_{50} values of ~ 600 μM (Fig. 2c).

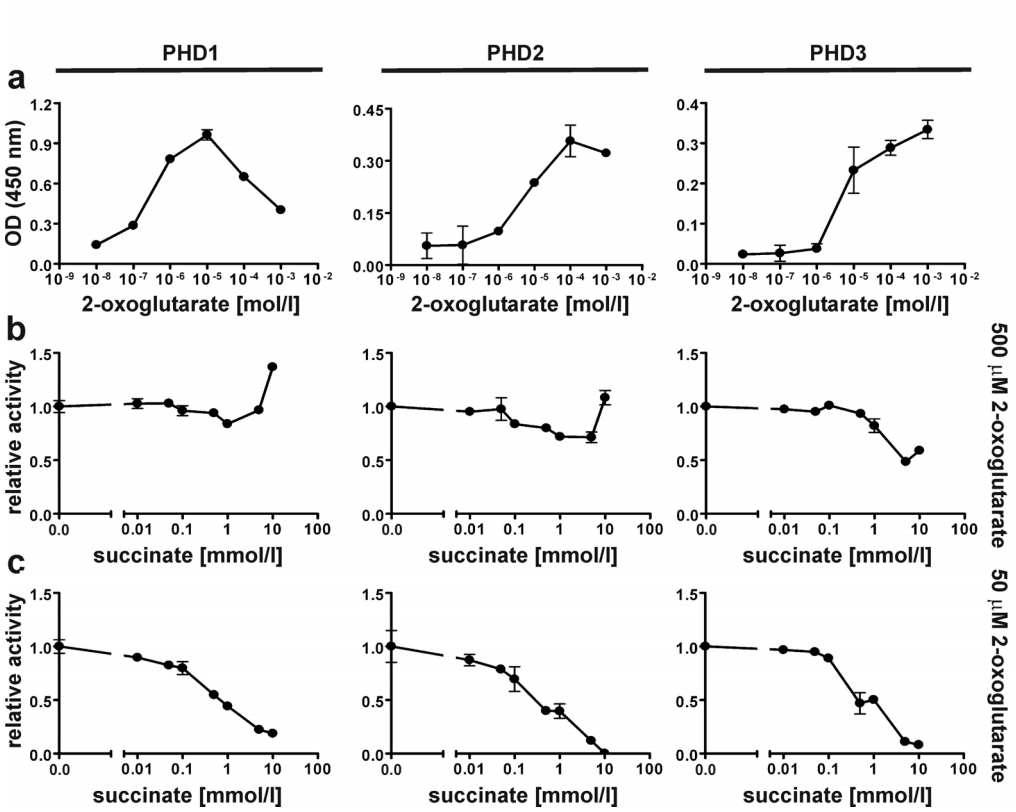

Fig. 2. Regulation of PHD function by Krebs cycle intermediates. PHD1, PHD2 and PHD3 (from left to right) hydroxylation activity was estimated in microtiter plate-based VBC binding assays. Titration curves of the co-substrate 2-oxoglutarate **(a)** or the co-product succinate in the presence of 500 µM **(b)** or 50 µM **(c)** 2-oxoglutarate are shown as mean values ± SEM of representative experiments performed in triplicates.

Vitamin C oxidation impairs PHD function

Vitamin C is essential for the function of collagen hydroxylases as well as for HIFα hydroxylases because it protects the enzyme's amino acid residues and/or active center iron from oxidation in reactions uncoupled from target hydroxylation (Myllylä et al., 1984). Purified PHDs do not show any activity in the absence of ascorbate and the addition of at least 0.1 mM ascorbate was required to fully induce hydroxylation activity of all three PHDs (Fig. 3a). The addition of oxidized ascorbate (dehydroascorbate) did not induce PHD activity (data not shown). These data confirm previously reported similar ascorbate Km values (140 - 180 μM) for all three PHDs (Hirsilä et al., 2003). Therefore, an excess of freshly prepared ascorbate (2 mM) was added to all subsequent experiments. Anti-oxidative ascorbate function is specifically required by the PHDs and cannot be replaced by the antioxidant N-acetyl-L-cysteine (Fig. 3b).

The absolute requirement for ascorbate might also explain the function of previously reported agents inhibiting PHD activity. Ascorbate oxidation by air can be measured by the drop in absorbance at 265 nm. However, as shown in Fig. 3c, ascorbate oxidation is negligible during the reaction period of 1 hour. Even in the presence of equimolar ferrous iron the ascorbate levels dropped only by 20% after 1 hour (Fig. 3c, left graph). The transition metals cobalt and nickel are well known inducers of HIFα protein stability due to PHD inhibition. Of note, even a two-fold molar excess of $CoCl_2$ or $NiSO_4$ did not catalyze more ascorbate oxidation than $FeSO_4$ (Fig. 3c, middle graph). In contrast, copper efficiently catalyzed ascorbate oxidation: 5 μM $CuCl_2$ destroyed more than 90% of 100 μM ascorbate within 10 minutes (Fig. 3c, right graph).

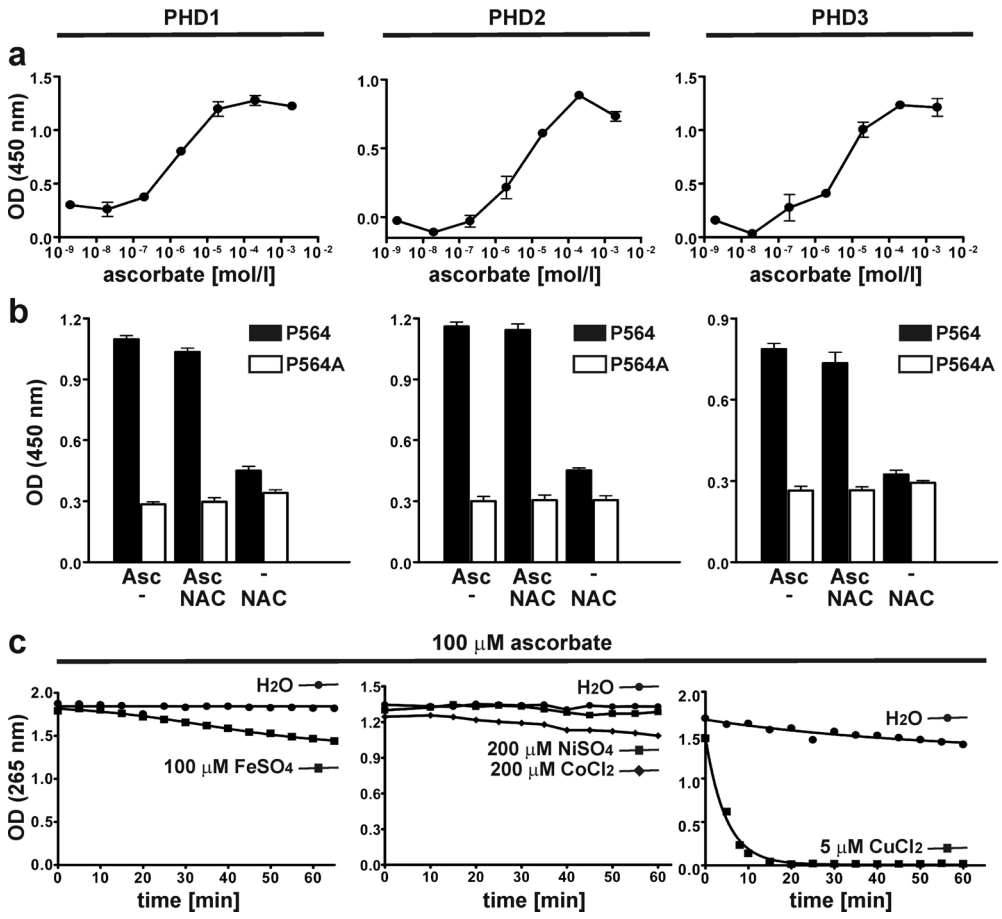

Fig. 3. Regulation of PHD function by vitamin C. PHD1, PHD2 and PHD3 (from left to right) hydroxylation activity was estimated in microtiter plate-based VBC binding assays. **(a)** Titration of vitamin C reveals that 0.1 mM ascorbate is minimally required for PHD function. **(b)** The anti-oxidant N-acetyl-L-cysteine (250 μM) cannot replace ascorbate (2 mM) for PHD function. **(c)** Ascorbate is slowly oxidized by air (dehydroascorbate does not absorb light at 265 nm). Ferrous iron slightly catalyzes this reaction (left graph), whereas the effects of the known PHD inhibitors cobalt and nickel are negligible (middle graph). However, cupric copper strongly catalyzed ascrobate oxidation (right graph). Mean values ± SEM of representative experiments performed in triplicates are shown.

The antioxidants gallic acid and n-propyl gallate efficiently inhibit the activity of all three PHDs

A number of natural nutrient compounds has been suggested to induce HIF-1α; among them flavonoid and non-flavonoid polyphenols. Catechins are non-flavonoid polyphenols found in green tea leaves which have been reported to induce HIF-1α (Zhou et al., 2004). Only catechins containing a 3-gallate moiety activate HIF-1 and have been shown to inhibit PHD2 activity (Thomas and Kim, 2005; Tsukiyama et al., 2006). Thus, we tested the sensitivity of PHD-dependent peptide hydroxylation on gallic acid or n-propyl gallate. Remarkably, all three PHDs were efficiently inhibited by 33 - 100 μM gallic acid (Fig. 4a) or by 3.3 - 10 μM n-propyl gallate (Fig. 4b). As shown in Fig. 4c, n-propyl gallate also induced HIF-dependent luciferase reporter gene activity in cultured CHO cells. In contrast to the *in vitro* results, gallic acid treatment of cells only marginally affected luciferase expression (data not shown).

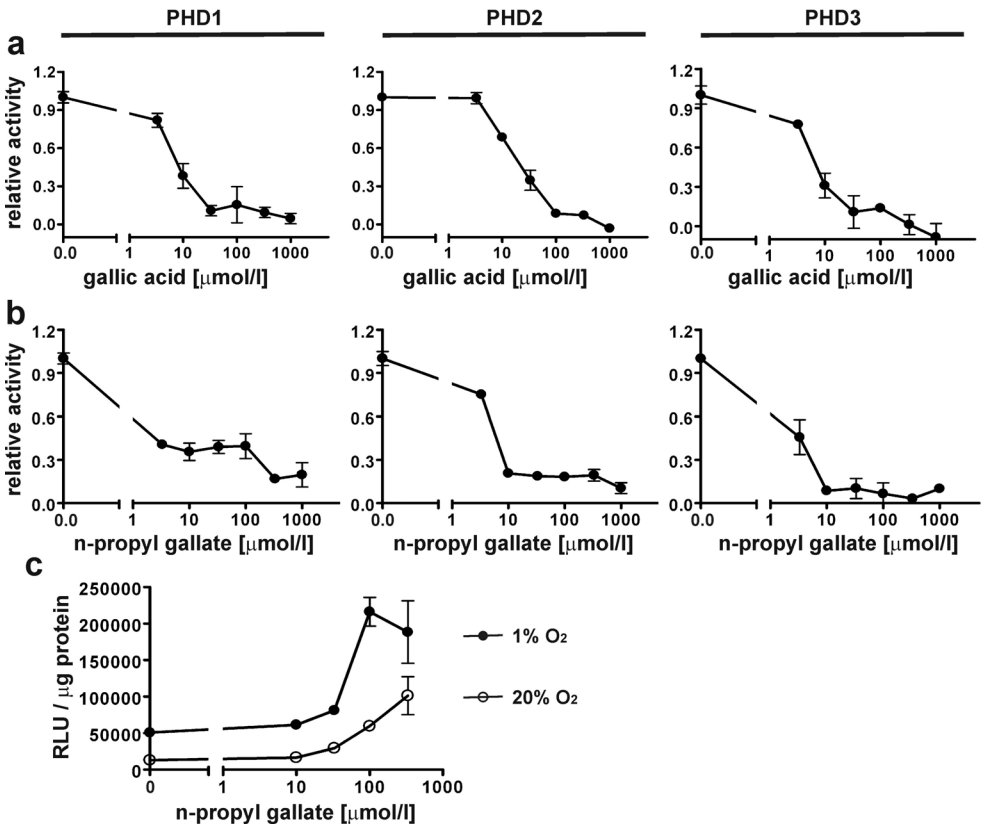

Fig. 4. Regulation of PHD function by gallate. The effects of gallic acid **(a)** and n-propyl gallate **(b)** on PHD1, PHD2 and PHD3 (from left to right) hydroxylation activity were estimated in microtiter plate-based VBC binding assays. **(c)** CHO cells stably transfected with a HIF-dependent luciferase reporter gene were treated with the indicated concentrations of n-propyl gallate for 24 hours. Relative light units (RLU) were normalized to the protein content of the lysates. Mean values ± SEM of representative experiments performed in triplicates (quadruplicates for the reporter gene assays) are shown.

Discussion

Oxygen sensing by protein hydroxylation is a regulated process

Sensing cellular pO_2 is probably not the only function of the PHDs. Otherwise, an excess of a single sensory protein simply regulated by oxygen availability would be all that was required for this task. However, there is ample evidence that the PHD oxygen sensors fulfill additional functions. First, different tissues are differently vascularized, unequally perfused and show spatially and temporally variable oxygen consumption rates. Thus, the mean pO_2 varies from tissue to tissue, if not from cell to cell. Nevertheless, every cell is capable of sensing a reduction in oxygenation and adequately responds to such a reduction by inducing HIF-dependent gene expression. Therefore, also hypoxia thresholds vary both spatially and temporally and the PHD oxygen sensors evolved to meet these requirements by showing a variable tissue-specific expression pattern. Second, every successful adaptation to hypoxia eventually results in re-oxygenation of the affected tissue. Because following hypoxia there are much higher levels of HIFα in the cell, the oxygen-dependent hydroxylation and degradation machinery must increase its capacity to cope with the degradation of the increased protein mass. Third, HIFα is also induced by a variety of stimuli under normoxic conditions. How can HIFα protein stabilization occur in the presence of active PHDs and sufficient oxygen supply? A plausible explanation lies in the limited expression levels of PHDs which are finely counterbalanced by HIFα levels. If either one is upregulated it overcomes the function of the other. Thus, as well as an increase in PHD synthesis leads to HIFα degradation, an increase in HIFα synthesis leads to its stabilization even under normoxic conditions. We have previously demonstrated that these mechanisms work even under hypoxic conditions, since PHD-dependent HIFα hydroxylation was reduced but still functional in direct proportion to the decrease in available oxygen (Stiehl et al., 2006).

Regulation of the three PHD oxygen sensors by small molecules

In this work, we have analyzed the effects of several small molecules on PHD function *in vitro*. Addition of ferrous iron was essential for PHD activity. Hirsilä *et al.* reported iron Km values for PHD1, PHD2 and PHD3 of 30 nM, 30 nM and 100 nM, respectively (Hirsila et al., 2005), and McNeill *et al.* reported a ferrous iron binding constant of highly purified PHD2 of << 1 μM (McNeill et al., 2005). Our data support

an increased iron affinity of PHD3 that reached maximal activity already in the presence of 1 μM $FeSO_4$ whereas PHD1 and PHD2 required 10 μM $FeSO_4$ for full activity. The iron chelator desferrioxamine efficiently inhibited PHD1 and PHD3 activity at concentrations corresponding to the iron concentrations in the reaction buffer. PHD2 inhibition was somewhat less efficient but a drop in activity was observed with similar desferrioxamine concentrations. Interestingly, it has previously been suggested that desferrioxamine inefficiently inhibited crude PHD enzymes *in vitro*: in the presence of 5 μM Fe^{2+} up to 1 mM desferrioxamine inhibited crude PHDs by < 20% whereas inhibition of pure PHDs was much more efficient (Hirsila et al., 2005). Thus, the efficiency of inhibition of the PHDs by desferrioxamine might allow to draw some conclusions on the purity of the enzyme preparation.

We previously determined an iron concentration in the FCS of 148 μM and in the cell culture medium of 16 μM (Wanner et al., 2000). The intracellular concentration of chelatable iron has been reported to be 3-6 μM, depending on the cell type (Rauen et al., 2000). Thus, the estimated concentration of "free" iron for optimal PHD activity *in vitro* corresponds to the iron concentrations *in vivo*, suggesting that iron availability is indeed able to regulate the HIF system by influencing PHD activity. This assumption has been confirmed by experimentally changing the "labile iron pool" in cancer cells (Jones et al., 2006; Knowles et al., 2006; Knowles et al., 2003). Interestingly, important iron uptake and transport proteins are among the targets of HIF-1 that might hence function also as a regulator of iron homeostasis in addition to oxygen homeostasis (Wenger et al., 2005). These values also explain why ~ 100 μM of the hexadentate extracellular iron chelator desferrioxamine but only ~ 10 μM of the bidentate intracellular iron chelator cyclopirox olamine were required to induce the HIF system by inhibiting PHD activity (Linden et al., 2003).

Krebs cycle intermediates such as succinate and fumarate are known to induce the HIF system by inhibiting PHD activity (Isaacs et al., 2005; Pollard et al., 2005; Selak et al., 2005). Moreover, 2-oxoacids such as pyruvate and oxaloacetate also induce HIFα (Dalgard et al., 2004; Lu et al., 2005). These findings link mitochondrial function and cellular metabolism with the PHD/HIF oxygen sensing system. Mutation of the tumor suppressor *succinate dehydrogenase* has been reported to increase cellular succinate concentration from 120 μM to ~ 440 μM (Selak et al., 2005). The IC_{50} values for succinate have been reported to be in the range of 510 - 830 μM for

purified PHDs (Koivunen et al., 2007). We obtained similar IC_{50} values (~ 600 μM) for succinate when the assays were performed in the presence of 50 μM 2-oxoglutarate. Succinate competes for 2-oxoglutarate binding to the PHDs (16, 26), explaining why we observed only weak succinate inhibition of PHD activity in the presence of high (500 μM) 2-oxoglutarate concentrations.

We demonstrated that ascorbate is essential for the function of all three PHDs. It has been shown that physiological concentrations (25 μM) of ascorbate suppress HIF-1α protein levels in cancer cells, suggesting that intracellular ascorbate concentrations indeed respresent a major regulator of PHD function (Knowles et al., 2003). Ascorbate depeletion thus could also explain how transition metals induce HIF-1α. Interestingly, Salnikow and colleagues reported that cellular ascorbate depletion causes $NiSO_4$ and $CoCl_2$ induced HIF-1α stabilization (Karaczyn et al., 2006; Salnikow et al., 2004). However, the authors attributed this effect to inhibition of cellular ascorbate uptake. Moreover, we could not observe any relevant nickel or cobalt-mediated degradation of ascorbate *in vitro*. In contrast, we observed a rapid copper-mediated ascorbate oxidation which most likely explains the previously reported $CuCl_2$ induced HIF-1α stabilization via PHD inhibition in cultured cells (Martin et al., 2005).

We have found that the antioxidant compounds gallic acid and n-propyl gallate efficiently inhibited all three PHDs *in vitro* and the esterified gallate also induced a HIF-dependent reporter gene in cell culture. Tsukiyama *et al.* hypothesized that two phenolate oxygen atoms of gallate chelate with the active center iron and the carboxyl group of gallate forms a strong ionic/hydrogen bonding with Arg383 of PHD2 (Tsukiyama et al., 2006). In the hands of these authors, n-propyl gallate was effective only in cell culture but not in cell-free PHD assays, which was attributed to the esterified carboxyl group of n-propyl gallate. However, in our hands n-propyl gallate was even more efficient in PHD inhibition *in vitro* than gallic acid. A likely reason for this discrepancy was the use of crude cell lysates in a 2-oxoglutarate conversion assay by these authors. We recently found that 2-oxoglutarate turnover using crude lysates is independent of PHD function (Wirthner R. et al., submitted for publication).

What might be the mechanism of PHD inhibition by gallate-containing antioxidants in comparision to other antioxidants such as N-acetyl-L-cysteine? The use of molecular dioxygen for oxidative decarboxylation-coupled protein

hydroxylation requires short-lived, highly reactive transition states of oxygen. Antioxidants fitting into the active center of PHDs might scavenge these ROS, thereby blocking the PHD reaction cycle. Similarly, exogenously produced ROS potentially could interfere with these reactive transition states of oxygen. It has been suggested that the increase in ROS content in *junD*$^{-/-}$ or mucin 1 (MUC1) knock-down cells leads to a decrease in PHD activity and hence to HIF-1α accumulation (Gerald et al., 2004; Yin et al., 2007). Thus, antioxidants paradoxically would have the potential to do both: protect PHD function from exogenous ROS and block PHD activity by destruction of PHD-inherent ROS intermediates. The effective function of a given antioxidant would depend on the accessibility to the active center, interference with transition metals and ascorbate, and the scavenging efficiency of mitochondrial and/or NADPH oxidase-derived ROS.

Regulation of the PHD oxygen sensors by other mechanisms

Up to date, only few reports deal with the regulation of PHDs by protein-protein interactions. The E3 ubiquitin ligase Siah2 regulates PHD1 and PHD3, but not PHD2, protein stability (Nakayama et al., 2004). PHD3, but not PHD1 or PHD2, appears to be a substrate for the TRiC chaperonin (Masson et al., 2004). OS-9 apparently is simultaneously interacting with both HIFα and PHD2 or PHD3, but not PHD1, thereby enhancing HIFα hydroxylation and degradation (Baek et al., 2005). MORG might provide the molecular scaffold for HIFα interaction specifically with PHD3 (Hopfer et al., 2006). Finally, we recently reported that FKBP38 specifically regulates the stability of PHD2 (Barth et al., 2007). These examples demonstrate two things: first, abundance and function of PHDs can also be regulated by specific proteins; and second, the three different PHDs are regulated in non-identical ways, further supporting their non-redundant role in oxygen sensing. While the cell-free *in vitro* assay used in this work provides useful informations on the regulated function of each PHD individually, it can of course not take into account the manifold additional modes of PHD regulation, e.g. by protein-protein interaction, found within a cell.

PHD2 and PHD3, but not PHD1 or FIH, are transcriptionally induced under hypoxic conditions (Aprelikova et al., 2004; Berra et al., 2003; Cioffi et al., 2003; D'Angelo et al., 2003; del Peso et al., 2003; Epstein et al., 2001; Marxsen et al., 2004). It could be shown that HIF is required for hypoxic induction of PHD2 and

PHD3 gene expression and hypoxia response elements were identified in the regulatory regions of the corresponding genes (Metzen et al., 2005; Pescador et al., 2005). Because the essential co-factor oxygen is basically lacking under hypoxic conditions, the HIF-dependent hypoxic increase in PHD abundance is somewhat paradoxical. It has been suggested that increased PHD levels accelerate the termination of the HIF response following re-oxygenation (Appelhoff et al., 2004; Aprelikova et al., 2004; Epstein et al., 2001; Marxsen et al., 2004). Indeed, biochemical *in vitro* studies revealed K_m values of purified PHDs for oxygen close to the pO_2 in air, suggesting that the kinetics of specific HIFα hydroxylation under hypoxic conditions are rather slow (Hirsilä et al., 2003). However, tissues *in situ* have to deal with a great variability of generally very low pO_2 values, even when the inspiratory pO_2 is considered to be "normoxic". Thus, the PHD oxygen sensors need to operate at different pO_2 setpoint values in different tissues. We recently demonstrated that a self-regulatory loop defines a specific threshold for HIFα-activation as a function of the actual pO_2 (Stiehl et al., 2006). As schematically outlined in Fig. 5, this negative feedback-loop includes: *i*) a HIF-dependent induction of PHD2 and PHD3 upon a reduction in oxygen supply; *ii*) a PHD-dependent, partial reduction of HIFα even under very low pO_2; *iii*) the subsequent partial reduction of the PHD2 and PHD3 levels; *iv*) the definition of a novel setpoint for oxygen sensing by leveling off the HIF/PHD ratio; *v*) a secondary response to a further, more severe hypoxic insult; and *vi*) the triggering by iron, ascorbate, Krebs cycle intermediates, ROS and antioxidants. Small molecule regulators of PHD activity are thus functionally relevant under normoxic as well as hypoxic conditions.

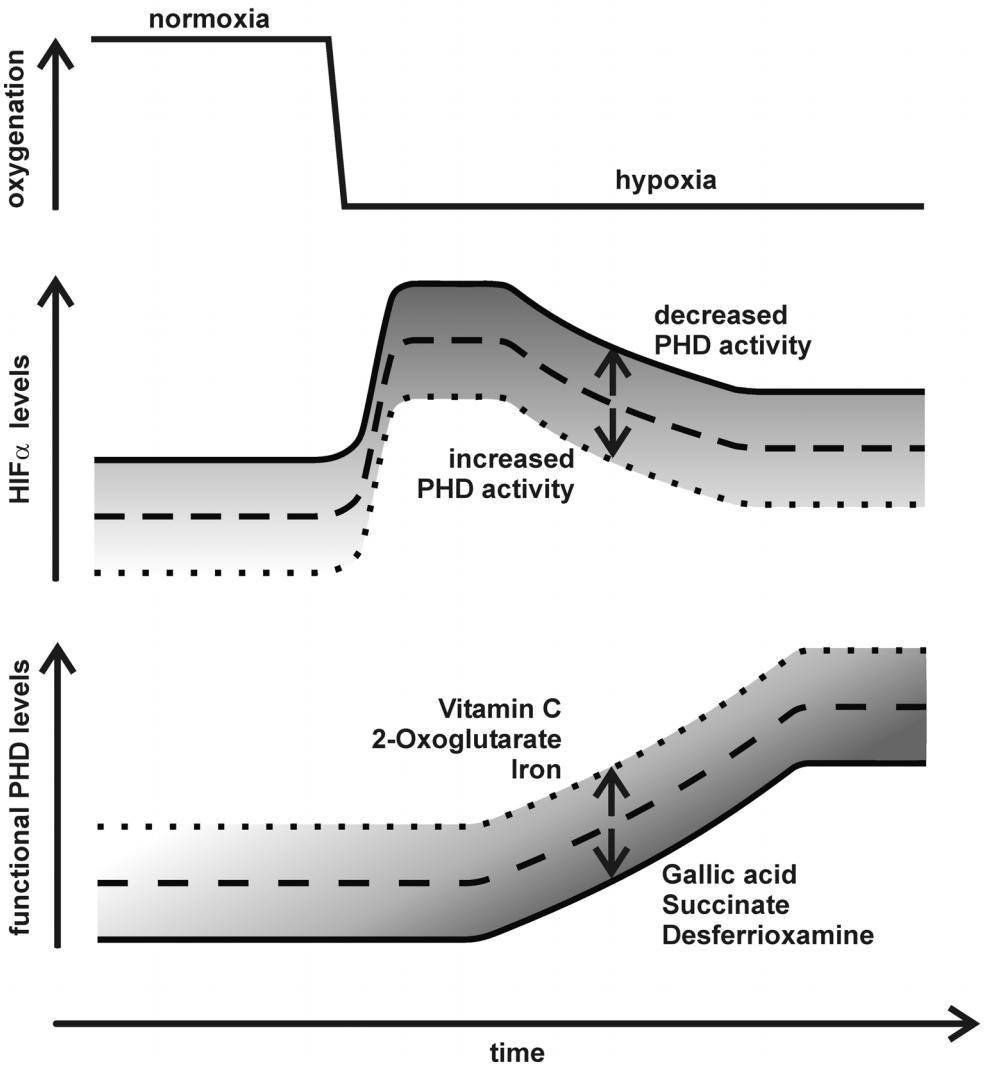

Fig. 5. Regulation of PHD-dependent oxygen sensing. Upon a hypoxic insult, the HIF-dependent transcriptional increase of PHD2 and PHD3 levels provides a negative feedback mechanism that defines a novel, adpated oxygen sensing setpoint capable of reacting to a second hypoxic insult. Small molecules contribute to the regulated oxygen sensing by triggering PHD function, leading to an inverse regulation of the HIF levels (for details see text).

Acknowledgments

The authors wish to thank S. Tan for providing the VBC expression vector. This work was supported by individual grants from the Forschungskredit of the University of Zürich (to D.P.S. and G.C.), Olga Mayenfisch-Stiftung (to G.C.), Sassella-Stifung (to D.P.S.), Krebsliga des Kantons Zürich (to R.H.W.), 6th Framework Programme of the European Commission/SBF (EUROXY LSHC-CT-2003-502932/SBF Nr. 03.0647-2 to R.H.W.) and Swiss National Science Foundation (3100A0-104219 to R.H.W. and G.C.).

Abbreviations

FIH, factor inhibiting HIF; HIF, hypoxia-inducible factor; Hyp, hydroxyproline; ODD, oxygen-dependent degradation; PHD, prolyl-4-hydroxylase domain protein; pO_2, oxygen partial pressure; pVHL, von Hippel-Lindau tumor suppressor protein; ROS, reactive oxygen species; VBC, pVHL/elongin B/elongin C.

References:

Abu-Farha M, Niles J, Willmore WG (2005). Erythroid-specific 5-aminolevulinate synthase protein is stabilized by low oxygen and proteasomal inhibition. Biochem Cell Biol. 83(5): 620-30.

Appelhoff RJ, Tian YM, Raval RR, Turley H, Harris AL, Pugh CW, Ratcliffe PJ, Gleadle JM (2004). Differential function of the prolyl hydroxylases PHD1, PHD2, and PHD3 in the regulation of hypoxia-inducible factor. J Biol Chem. 279(37): 38458-65.

Aprelikova O, Chandramouli GV, Wood M, Vasselli JR, Riss J, Maranchie JK, Linehan WM, Barrett JC (2004). Regulation of HIF prolyl hydroxylases by hypoxia-inducible factors. J Cell Biochem. 92(3): 491-501.

Baek JH, Mahon PC, Oh J, Kelly B, Krishnamachary B, Pearson M, Chan DA, Giaccia AJ, Semenza GL (2005). OS-9 interacts with hypoxia-inducible factor 1alpha and prolyl hydroxylases to promote oxygen-dependent degradation of HIF-1alpha. Mol Cell. 17(4): 503-12.

Barth S, Nesper J, Hasgall PA, Wirthner R, Nytko KJ, Edlich F, Katschinski DM, Stiehl DP, Wenger RH, Camenisch G (2007). The peptidyl prolyl cis/trans isomerase FKBP38 determines hypoxia-inducible transcription factor prolyl-4-hydroxylase PHD2 protein stability. Mol Cell Biol. 27(10): 3758-68.

Berra E, Benizri E, Ginouvés A, Volmat V, Roux D, Pouysségur J (2003). HIF prolyl-hydroxylase 2 is the key oxygen sensor setting low steady-state levels of HIF-1α in normoxia. EMBO J 22(16): 4082-90.

Bruick RK, McKnight SL (2001). A conserved family of prolyl-4-hydroxylases that modify HIF. Science 294(5545): 1337-40.

Cioffi CL, Liu XQ, Kosinski PA, Garay M, Bowen BR (2003). Differential regulation of HIF-1 alpha prolyl-4-hydroxylase genes by hypoxia in human cardiovascular cells. Biochem Biophys Res Commun. 303(3): 947-53.

Cockman ME, Masson N, Mole DR, Jaakkola P, Chang GW, Clifford SC, Maher ER, Pugh CW, Ratcliffe PJ, Maxwell PH (2000). Hypoxia inducible factor-alpha binding and ubiquitylation by the von Hippel-Lindau tumor suppressor protein. J Biol Chem. 275(33): 25733-41.

Cummins EP, Berra E, Comerford KM, Ginouves A, Fitzgerald KT, Seeballuck F, Godson C, Nielsen JE, Moynagh P, Pouyssegur J, Taylor CT (2006). Prolyl hydroxylase-1 negatively regulates IkappaB kinase-beta, giving insight into hypoxia-induced NFkappaB activity. Proc Natl Acad Sci U S A. 103(48): 18154-9.

D'Angelo G, Duplan E, Boyer N, Vigne P, Frelin C (2003). Hypoxia up-regulates prolyl hydroxylase activity: a feedback mechanism that limits HIF-1 responses during reoxygenation. J Biol Chem. 278(40): 38183-7.

Dalgard CL, Lu H, Mohyeldin A, Verma A (2004). Endogenous 2-oxoacids differentially regulate expression of oxygen sensors. Biochem J. 380(Pt 2): 419-24.

del Peso L, Castellanos MC, Temes E, Martin-Puig S, Cuevas Y, Olmos G, Landazuri MO (2003). The von Hippel Lindau/hypoxia-inducible factor (HIF) pathway regulates the transcription of the HIF-proline hydroxylase genes in response to low oxygen. J Biol Chem. 278(49): 48690-5.

Epstein AC, Gleadle JM, McNeill LA, Hewitson KS, O'Rourke J, Mole DR, Mukherji M, Metzen E, Wilson MI, Dhanda A, Tian YM, Masson N, Hamilton DL, Jaakkola P, Barstead R, Hodgkin J, Maxwell PH, Pugh CW, Schofield CJ,

Ratcliffe PJ (2001). C. elegans EGL-9 and mammalian homologs define a family of dioxygenases that regulate HIF by prolyl hydroxylation. Cell. 107(1): 43-54.

Gerald D, Berra E, Frapart YM, Chan DA, Giaccia AJ, Mansuy D, Pouysségur J, Yaniv M, Mechta-Grigoriou F (2004). JunD reduces tumor angiogenesis by protecting cells from oxidative stress. Cell. 118(6): 781-94.

Hirsilä M, Koivunen P, Günzler V, Kivirikko KI, Myllyharju J (2003). Characterization of the human prolyl 4-hydroxylases that modify the hypoxia-inducible factor. J Biol Chem 278(33): 30772-80.

Hirsila M, Koivunen P, Xu L, Seeley T, Kivirikko KI, Myllyharju J (2005). Effect of desferrioxamine and metals on the hydroxylases in the oxygen sensing pathway. Faseb J. 19(10): 1308-10.

Hopfer U, Hopfer H, Jablonski K, Stahl RA, Wolf G (2006). The novel WD-repeat protein Morg1 acts as a molecular scaffold for hypoxia-inducible factor prolyl hydroxylase 3 (PHD3). J Biol Chem. 281(13): 8645-55.

Isaacs JS, Jung YJ, Mole DR, Lee S, Torres-Cabala C, Chung YL, Merino M, Trepel J, Zbar B, Toro J, Ratcliffe PJ, Linehan WM, Neckers L (2005). HIF overexpression correlates with biallelic loss of fumarate hydratase in renal cancer: novel role of fumarate in regulation of HIF stability. Cancer Cell. 8(2): 143-53.

Jewell UR, Kvietikova I, Scheid A, Bauer C, Wenger RH, Gassmann M (2001). Induction of HIF-1alpha in response to hypoxia is instantaneous. Faseb J. 15(7): 1312-4.

Jones DT, Trowbridge IS, Harris AL (2006). Effects of transferrin receptor blockade on cancer cell proliferation and hypoxia-inducible factor function and their differential regulation by ascorbate. Cancer Res. 66(5): 2749-56.

Karaczyn A, Ivanov S, Reynolds M, Zhitkovich A, Kasprzak KS, Salnikow K (2006). Ascorbate depletion mediates up-regulation of hypoxia-associated proteins by cell density and nickel. J Cell Biochem. 97(5): 1025-35.

Knowles HJ, Mole DR, Ratcliffe PJ, Harris AL (2006). Normoxic stabilization of hypoxia-inducible factor-1alpha by modulation of the labile iron pool in differentiating U937 macrophages: effect of natural resistance-associated macrophage protein 1. Cancer Res. 66(5): 2600-7.

Knowles HJ, Raval RR, Harris AL, Ratcliffe PJ (2003). Effect of ascorbate on the activity of hypoxia-inducible factor in cancer cells. Cancer Res 63(8): 1764-8.

Koivunen P, Hirsila M, Remes AM, Hassinen IE, Kivirikko KI, Myllyharju J (2007). Inhibition of hypoxia-inducible factor (HIF) hydroxylases by citric acid cycle intermediates: possible links between cell metabolism and stabilization of HIF. J Biol Chem. 282(7): 4524-32.

Kuznetsova AV, Meller J, Schnell PO, Nash JA, Ignacak ML, Sanchez Y, Conaway JW, Conaway RC, Czyzyk-Krzeska MF (2003). von Hippel-Lindau protein binds hyperphosphorylated large subunit of RNA polymerase II through a proline hydroxylation motif and targets it for ubiquitination. Proc Natl Acad Sci U S A. 100(5): 2706-11.

Lando D, Peet DJ, Gorman JJ, Whelan DA, Whitelaw ML, Bruick RK (2002). FIH-1 is an asparaginyl hydroxylase enzyme that regulates the transcriptional activity of hypoxia-inducible factor. Genes Dev. 16(12): 1466-71.

Linden T, Katschinski DM, Eckhardt K, Scheid A, Pagel H, Wenger RH (2003). The antimycotic ciclopirox olamine induces HIF-1alpha stability, VEGF expression, and angiogenesis. Faseb J. 17(6): 761-3.

Lu H, Dalgard CL, Mohyeldin A, McFate T, Tait AS, Verma A (2005). Reversible inactivation of HIF-1 prolyl hydroxylases allows cell metabolism to control basal HIF-1. J Biol Chem 280(51): 41928-39.

Mahon PC, Hirota K, Semenza GL (2001). FIH-1: a novel protein that interacts with HIF-1alpha and VHL to mediate repression of HIF-1 transcriptional activity. Genes Dev. 15(20): 2675-86.

Martin F, Linden T, Katschinski DM, Oehme F, Flamme I, Mukhopadhyay CK, Eckhardt K, Troger J, Barth S, Camenisch G, Wenger RH (2005). Copper-dependent activation of hypoxia-inducible factor (HIF)-1: implications for ceruloplasmin regulation. Blood. 105(12): 4613-9.

Marxsen JH, Stengel P, Doege K, Heikkinen P, Jokilehto T, Wagner T, Jelkmann W, Jaakkola P, Metzen E (2004). Hypoxia-inducible factor-1 (HIF-1) promotes its degradation by induction of HIF-alpha-prolyl-4-hydroxylases. Biochem J. 381(Pt 3): 761-7.

Masson N, Appelhoff RJ, Tuckerman JR, Tian YM, Demol H, Puype M, Vandekerckhove J, Ratcliffe PJ, Pugh CW (2004). The HIF prolyl hydroxylase PHD3 is a potential substrate of the TRiC chaperonin. FEBS Lett. 570(1-3): 166-70.

Maxwell PH, Wiesener MS, Chang GW, Clifford SC, Vaux EC, Cockman ME, Wykoff CC, Pugh CW, Maher ER, Ratcliffe PJ (1999). The tumour suppressor protein VHL targets hypoxia-inducible factors for oxygen-dependent proteolysis. Nature. 399(6733): 271-5.

McNeill LA, Flashman E, Buck MR, Hewitson KS, Clifton IJ, Jeschke G, Claridge TD, Ehrismann D, Oldham NJ, Schofield CJ (2005). Hypoxia-inducible factor prolyl hydroxylase 2 has a high affinity for ferrous iron and 2-oxoglutarate. Mol Biosyst 1(4): 321-4.

Metzen E, Stiehl DP, Doege K, Marxsen JH, Hellwig-Burgel T, Jelkmann W (2005). Regulation of the prolyl hydroxylase domain protein 2 (phd2/egln-1) gene: identification of a functional hypoxia-responsive element. Biochem J. 387(Pt 3): 711-7.

Myllylä R, Majamaa K, Günzler V, Hanauske-Abel HM, Kivirikko KI (1984). Ascorbate is consumed stoichiometrically in the uncoupled reactions catalyzed by prolyl 4-hydroxylase and lysyl hydroxylase. J Biol Chem 259(9): 5403-5.

Nakayama K, Frew IJ, Hagensen M, Skals M, Habelhah H, Bhoumik A, Kadoya T, Erdjument-Bromage H, Tempst P, Frappell PB, Bowtell DD, Ronai Z (2004). Siah2 regulates stability of prolyl-hydroxylases, controls HIF1alpha abundance, and modulates physiological responses to hypoxia. Cell. 117(7): 941-52.

Oehme F, Ellinghaus P, Kolkhof P, Smith TJ, Ramakrishnan S, Hutter J, Schramm M, Flamme I (2002). Overexpression of PH-4, a novel putative proline 4-hydroxylase, modulates activity of hypoxia-inducible transcription factors. Biochem Biophys Res Commun. 296(2): 343-9.

Oehme F, Jonghaus W, Narouz-Ott L, Huetter J, Flamme I (2004). A nonradioactive 96-well plate assay for the detection of hypoxia-inducible factor prolyl hydroxylase activity. Anal Biochem. 330(1): 74-80.

Pescador N, Cuevas Y, Naranjo S, Alcaide M, Villar D, Landazuri MO, Del Peso L (2005). Identification of a functional hypoxia-responsive element that regulates the expression of the egl nine homologue 3 (egln3/phd3) gene. Biochem J. 390(Pt 1): 189-97.

Pollard PJ, Briere JJ, Alam NA, Barwell J, Barclay E, Wortham NC, Hunt T, Mitchell M, Olpin S, Moat SJ, Hargreaves IP, Heales SJ, Chung YL, Griffiths JR, Dalgleish A, McGrath JA, Gleeson MJ, Hodgson SV, Poulsom R, Rustin P, Tomlinson IP (2005). Accumulation of Krebs cycle intermediates and over-expression of HIF1alpha in tumours which result from germline FH and SDH mutations. Hum Mol Genet. 14(15): 2231-9.

Pouyssegur J, Dayan F, Mazure NM (2006). Hypoxia signalling in cancer and approaches to enforce tumour regression. Nature. 441(7092): 437-43.

Rauen U, Petrat F, Li T, De Groot H (2000). Hypothermia injury/cold-induced apoptosis--evidence of an increase in chelatable iron causing oxidative injury in spite of low O2-/H2O2 formation. Faseb J. 14(13): 1953-64.

Salnikow K, Donald SP, Bruick RK, Zhitkovich A, Phang JM, Kasprzak KS (2004). Depletion of intracellular ascorbate by the carcinogenic metals nickel and cobalt results in the induction of hypoxic stress. J Biol Chem 279(39): 40337-44.

Schofield CJ, Ratcliffe PJ (2004). Oxygen sensing by HIF hydroxylases. Nat Rev Mol Cell Biol. 5(5): 343-54.

Selak MA, Armour SM, MacKenzie ED, Boulahbel H, Watson DG, Mansfield KD, Pan Y, Simon MC, Thompson CB, Gottlieb E (2005). Succinate links TCA cycle dysfunction to oncogenesis by inhibiting HIF-alpha prolyl hydroxylase. Cancer Cell. 7(1): 77-85.

Stait SE, Leake DS (1996). The effects of ascorbate and dehydroascorbate on the oxidation of low-density lipoprotein. Biochem J. 320(Pt 2): 373-81.

Stiehl DP, Wirthner R, Köditz J, Spielmann P, Camenisch G, Wenger RH (2006). Increased prolyl 4-hydroxylase domain proteins compensate for decreased oxygen levels. Evidence for an autoregulatory oxygen-sensing system. J Biol Chem 281(33): 23482-91.

Takeda K, Ho VC, Takeda H, Duan LJ, Nagy A, Fong GH (2006). Placental but not heart defects are associated with elevated hypoxia-inducible factor α levels in mice lacking prolyl hydroxylase domain protein 2. Mol Cell Biol 26(22): 8336-46.

Thomas R, Kim MH (2005). Epigallocatechin gallate inhibits HIF-1alpha degradation in prostate cancer cells. Biochem Biophys Res Commun. 334(2): 543-8.

Tsukiyama F, Nakai Y, Yoshida M, Tokuhara T, Hirota K, Sakai A, Hayashi H, Katsumata T (2006). Gallate, the component of HIF-inducing catechins, inhibits HIF prolyl hydroxylase. Biochem Biophys Res Commun. 351(1): 234-9.

Wang J, Chen G, Muckenthaler M, Galy B, Hentze MW, Pantopoulos K (2004). Iron-mediated degradation of IRP2, an unexpected pathway involving a 2-oxoglutarate-dependent oxygenase activity. Mol Cell Biol. 24(3): 954-65.

Wang J, Pantopoulos K (2005). The pathway for IRP2 degradation involving 2-oxoglutarate-dependent oxygenase(s) does not require the E3 ubiquitin ligase activity of pVHL. Biochim Biophys Acta. 1743(1-2): 79-85.

Wanner RM, Spielmann P, Stroka DM, Camenisch G, Camenisch I, Scheid A, Houck DR, Bauer C, Gassmann M, Wenger RH (2000). Epolones induce erythropoietin expression via hypoxia-inducible factor-1 alpha activation. Blood. 96(4): 1558-65.

Wenger RH (2002). Cellular adaptation to hypoxia: O2-sensing protein hydroxylases, hypoxia-inducible transcription factors, and O2-regulated gene expression. Faseb J. 16(10): 1151-62.

Wenger RH, Stiehl DP, Camenisch G (2005). Integration of oxygen signaling at the consensus HRE. Sci STKE 2005(306): re12.

Yin L, Kharbanda S, Kufe D (2007). Mucin 1 oncoprotein blocks hypoxia-inducible factor 1alpha activation in a survival response to hypoxia. J Biol Chem. 282(1): 257-66.

Zhou YD, Kim YP, Li XC, Baerson SR, Agarwal AK, Hodges TW, Ferreira D, Nagle DG (2004). Hypoxia-inducible factor-1 activation by (-)-epicatechin gallate: potential adverse effects of cancer chemoprevention with high-dose green tea extracts. J Nat Prod. 67(12): 2063-9.

Author Manuscript II published in Blood, 2011 Feb 23. [Epub ahead of print].
PMID: 21346252

7. Manuscript II: Vitamin C is dispensable for oxygen sensing *in vivo*

Katarzyna J. Nytko[1], Nobuyo Maeda[2], Philipp Schläfli[1], Patrick Spielmann[1], Roland H. Wenger[1], and Daniel P. Stiehl[1]

[1]Department of Physiology and Zürich Center for Integrative Human Physiology ZIHP, University of Zürich, CH-8057 Zürich, Switzerland; [2]Department of Pathology and Laboratory of Medicine, University of North Carolina at Chapel Hill, Chapel Hill, NC 27599, USA

Running Title:
REDOX RESPONSE OF OXYGEN SENSING PHDs

Correspondence:
Daniel P. Stiehl, M.D.
Institute of Physiology
University of Zürich-Irchel
Winterthurerstr. 190
CH-8057 Zürich, Switzerland
phone: +41 44 6355090
fax: +41 44 6356814
email: daniel.stiehl@access.uzh.ch

Author Manuscript II published in Blood, 2011 Feb 23. [Epub ahead of print].
PMID: 21346252

Abstract

Prolyl-4-hydroxylation is necessary for proper structural assembly of collagens and oxygen-dependent protein stability of hypoxia-inducible transcription factors (HIFs). *In vitro* function of HIF prolyl-4-hydroxylase domain (PHD) enzymes requires oxygen and 2-oxoglutarate as cosubstrates with iron (II) and vitamin C serving as co-factors. While vitamin C deficiency is known to cause the collagen-disassembly disease scurvy, it is unclear whether cellular oxygen sensing is similarly affected. Here we report that vitamin C deprived $Gulo^{-/-}$ knock-out mice show normal HIF-dependent gene expression. The systemic response of $Gulo^{-/-}$ animals to inspiratory hypoxia, as measured by plasma erythropoietin levels, was similar to animals supplemented with vitamin C. Hypoxic HIF induction was also essentially normal under serum and vitamin C-free cell culture conditions, suggesting that vitamin C is not required for oxygen sensing *in vivo*. Glutathione was found to fully substitute for vitamin C requirement of all three PHD isoforms *in vitro*. Consistently, glutathione also reduced HIF-1α protein levels, transactivation activity and endogenous target gene expression in cells exposed to $CoCl_2$. A C201S mutation in PHD2 increased basal hydroxylation rates and conferred resistance to oxidative damage *in vitro*, suggesting that this surface accessible PHD2 cysteine residue is a target of antioxidative protection by vitamin C and glutathione.

Introduction

Oxygen is essential for a number of physiological processes, particularly for cellular respiration and energy metabolism. On the molecular level, response to hypoxia is mediated by hypoxia-inducible transcription factors termed HIFs. Under continuous oxygen supply, two distinct prolyl residues within the oxygen-dependent degradation domain (ODD) of HIFα subunits are hydroxylated by prolyl-4-hydroxylase domain-containing enzymes (PHDs). Hydroxy-HIFα is recognized by the von Hippel-Lindau tumor suppressor protein (pVHL) and subsequently targeted for proteasomal degradation (Ivan et al., 2001; Jaakkola et al., 2001). When oxygen is limited, PHD activity ceases, non-hydroxylated HIFα is stabilized and heterodimerizes with the HIFβ subunit to activate expression of numerous target genes (Wenger et al., 2005). Moreover, an asparaginyl hydroxylase termed factor inhibiting HIF (FIH) hydroxylates a C-terminal Asn residue of HIF-α subunits in an oxygen-dependent manner, thereby regulation co-factor recruitment and HIF's transcriptional activity (Hewitson et al., 2002).

Three PHD isoforms have been characterized so far, termed PHD1, PHD2 and PHD3, which differ in size, subcellular localization and tissue distribution (Kaelin and Ratcliffe, 2008). PHD2 is the most ubiquitously expressed isoform, responsible for the normoxic control of HIFα (Berra et al., 2003). Accordingly, genetic ablation of PHD2 but not PHD1 or PHD3 results in embryonic lethality in mice (Takeda et al., 2006). Suggesting a fundamental role in the hematopoietic and circulatory systems, somatic inactivation of PHD2 leads to increased erythropoiesis and angiogenesis as a result of HIFα stabilization followed by activation of its target genes, including erythropoietin (EPO) and vascular endothelial growth factor (VEGF) (Takeda et al., 2007). Knock-out of either PHD1 or PHD3 had no effect on hematological parameters. However, combined PHD1/PHD3 knock-out animals showed a slight increase in hematocrit, hemoglobin and red blood cell counts (Takeda et al., 2008). Clinical data on patients with erythrocytosis revealed P317R or R371H mutations in the gene encoding for PHD2, altering the hydroxylation efficiency of the mutant protein (Percy et al., 2007; Percy et al., 2006). A third point mutation in PHD2 (H374R) was found in a patient suffering from erythrocytosis and paraganglioma (Ladroue et al., 2008). These case reports emphasize the critical role of PHD2 in regulating erythropoiesis and maintaining red blood cell homeostasis also in humans.

PHDs belong to a larger superfamily of 2-oxoglutarate and Fe(II)-dependent di-oxygenases. Similar to collagen prolyl-4-hydroxylase (C-P4H, EC 1.14.11.2), PHDs require molecular oxygen and 2-oxoglutarate as co-substrates, as well as ferrous iron and probably vitamin C as co-factors for enzymatic activity (Hirsilä et al., 2003). K_m-values of PHDs for oxygen are strikingly higher than those of other prolyl-4-hydroxylases (Hirsilä et al., 2003). The relatively low oxygen affinity is essential for effective oxygen sensing, since even small changes in oxygen partial pressure can influence hydroxylation activity (Flashman et al., 2010; Hirsilä et al., 2003; Stiehl et al., 2006).

In a previous study we reported on the dose-dependent regulation of the *in vitro* activity of all three PHD isoforms by their essential co-substrates and co-factors, including vitamin C (Nytko et al., 2007). Primates, including humans, lost the ability to *de novo* synthesize vitamin C and thus depend on dietary vitamin C intake. Since ascorbate is an essential co-factor for C-P4Hs which hydroxylate proline residues to stabilize the collagen triple helix structure, persistent ascorbate deficiency results in disassembly of connective tissue structures, a common symptom of the nowadays rare disease scurvy (Mandl et al., 2009). With K_m-values ranging from 140-180 μM, the requirement of PHDs for vitamin C *in vitro* is only two fold lower than for C-P4H, suggesting that also HIF hydroxylases could well be affected by vitamin C malnutrition (Hirsilä et al., 2003). Mice lacking a functional *Gulo* gene have been described as a model to study vitamin C deficiency (Maeda et al., 2000). *Gulo* encodes for L-gulono-1,4-lactone-oxidase (GLO, EC 1.1.3.8), a key enzyme involved in the final step of L-ascorbic acid (vitamin C) biosynthesis. Dietary vitamin C deprivation leads to body weight loss, anemia, aortic wall damage and internal hemorrhages in these mice (Maeda et al., 2000).

While the interaction between the target prolyl residue, molecular oxygen, 2-oxoglutarate and iron during the reaction cycle in the active center of PHDs has been described in detail, the apparently inevitable presence of vitamin C for the *in vitro* function of the PHDs remains elusive (Bruick and McKnight, 2001; Chowdhury et al., 2009). Due to its antioxidative properties, vitamin C might maintain ferrous iron in the reduced state. Given the enzymatic relationship between HIFα and collagen prolyl-4-hydroxylases, we set out to investigate the effect of dietary vitamin C on the

regulation of the PHD-HIF oxygen sensing pathway in Gulo$^{-/-}$ mice under normoxaemic and hypoxaemic conditions.

Materials and methods

Cell culture. HeLa human cervix carcinoma cells were adapted to Ham´s Nutrient Mixture F12 (Sigma), free of ascorbate and FCS, containing the following supplements: EGF (50 ng/ml, Sigma), insulin (5 μg/ml, Sigma), apo-transferrin (5 μg/ml, Sigma), hydrocortisone (100 nM, Sigma) buffered with 15 mM Hepes (pH 7.4) as well as 100 IU/ml penicillin and 100 μg/ml streptomycin. HepG2 human hepatoma cells stably transfected with a HIF-dependent firefly luciferase reporter gene termed HRG1 have been described before (Stiehl et al., 2002). If not indicated otherwise, all cells were maintained in Dulbecco's Modified Eagle Medium with 10% FCS, 100 IU/ml penicillin and 100 μg/ml streptomycin. Cell number and viability was determined using a ViCell Counter (Beckman).

HIF transactivation activity. 5×10^5 HeLa cells were co-transfected with 500 ng of the HIF-dependent pH3SVL reporter vector containing a total of 6 HIF binding sites derived from regulatory elements of the *transferrin* gene (Wanner et al., 2000) and 40 ng of pRL-CMV *Renilla* luciferase expression plasmid (Promega) essentially as described before (Stiehl et al., 2006). 24 hours post transfection, cells were split and exposed to graded oxygen concentrations (21%-0.2% oxygen) for 24 hours using cross-calibrated oxygen-controlled CO_2 incubators (Binder CB 150). Stably transfected HRG1 HIF reporter cells were adapted to 1% FCS overnight and treated with 50 μM desferrioxamine mesylate (DFX, Sigma) or 100 μM $CoCl_2$ and 1-10 mM reduced glutathione (GSH, 250 mM stock solution adjusted to pH 7.0) or 0.2-2 mM ascorbate for 24 hours. For hypoxic experiments, cells were grown under 2% O_2 for 24 hours and treated with GSH or ascorbate. HRG1 cells were transfected with pRL-SV40 *Renilla* luciferase to control for non-HIF mediated effects of ascorbate and GSH on the heterologous SV40 minimal promoter present in both constructs. Cells were lysed using passive lysis buffer and luciferase activities were determined according to the manufacturer's instructions (Promega) using a 96-well luminometer (Berthold). Data are expressed as relative luciferase activities per total cellular protein of experiments performed in triplicates by calculating the ratio of *firefly/renilla* activities per well.

Expression and purification of recombinant PHD enzymes. Recombinant PHD proteins were expressed and purified as glutathione-S-transferase (GST)-fusion proteins from baculovirus-infected Sf9 insect cells as described earlier (Stiehl et al., 2006). Untagged enzyme preparations were obtained by introducing a PreScission protease cleavage site between the GST-tag and the PHD open reading frame. A C201S point mutation was introduced into the human PHD2 expression plasmid by site-directed mutagenesis (Stratagene). Untagged PHD2 was expressed in Sf9 cells and purified by conventional ion-exchange chromatography (kind gift of Dr. Felix Oehme, Bayer Healthcare, Wuppertal, Germany). Purity of the enzyme preparations was analyzed by SDS-PAGE followed by Coomassie staining or immunoblotting.

Prolyl-4-hydroxylation assay. Activity of recombinant PHD enzymes was measured by a microtiter plate-based peptide hydroxylation assay as described before (Wirthner et al., 2007). Briefly, recombinant PHDs were used to hydroxylate a biotinylated peptide derived from HIF-1α (amino acid residues 556 to 574) coupled to streptavidin-coated 96-well plates. Hydroxylation reaction was performed for 1 hour at room temperature in the presence of 10 μM $FeSO_4$, 0.5 mM 2-oxoglutarate and 2 mM ascorbate in 20 mM Tris-HCl pH 7.5, 5 mM KCl and 1.5 mM $MgCl_2$. Hydroxylated peptides were detected by recombinant, thioredoxin-tagged von Hippel-Lindau/elongin B/elongin C (VBC) complex. Reactions were stopped by removing the reaction mix and adding 1 mM H_2O_2. Bound VBC complex was detected by rabbit anti-thioredoxin antibodies and secondary HRP-conjugated anti-rabbit antibodies (Sigma) using the 3,3,5,5-tetramethylbenzidine substrate kit (Pierce). The peroxidase reaction was stopped by adding 2 M H_2SO_4 and absorbance was determined at 450 nm in a microplate reader. Background values as determined by using a mutant HIF-1α (P564A) peptide were subtracted for each experiment.

Ascorbate determination. Ascorbate content of *in vitro* hydroxylation assay samples was quantified by HPLC as described before (Simoes et al., 2003). Briefly, a 10-fold dilution of the enzyme reaction mix containing 2 mM ascorbate was analyzed before and after 1 hour of hydroxylation reaction. Following dilution in the mobile phase (60 mM phosphoric acid, pH 3.1), a 20 μl sample was injected onto a Nucleosil C18 column and eluted applying an acetonitrile gradient (0-60%). Ascorbate elution

was monitored at 254 nm, corresponding to 96% absorbance of ascorbate and only 4% of dehydroascorbate (Mody et al., 2005). Chromatograms and standard curve of pure ascorbate ranging from 25-200 μM were used to calculate the content of ascorbate in study samples (Supplementary Fig. 2). Ascorbate levels in plasma samples of mice were determined by HPLC (Swiss Vitamin Institute, Epalinges, Switzerland).

Immunoblot analyses. Total soluble cellular proteins were extracted with a high salt extraction buffer containing 0.4 M NaCl, 0.1% Nonidet P-40, 10 mM Tris-HCl (pH 8.0), 1 mM EDTA, 1 mM dithiothreitol, 1 mM phenylmethylsulfonyl fluoride and protease inhibitor cocktail (Sigma). Protein concentration was measured by the method of Bradford and 60-70 μg of cellular protein was subjected to immunoblot analyses. Membranes were probed using the following dilutions of mouse monoclonal (mAb) or rabbit polyclonal antibodies: mAb anti-HIF-1α (BD Transduction Laboratories, 1:1000), mAb anti-CA9 (M75, kindly provided by S. Pastorekova, Bratislava, Slovak Republic), mAb anti-β-actin (Sigma, 1:5000), rabbit anti-PHD1 (Genway, 1:2000), rabbit anti-PHD2 (Novus, 1:1000) or rabbit anti-PHD3 (Novus, 1:1000), followed by secondary HRP-conjugated antibodies (all Pierce, 1:2000).

mRNA quantification. Total RNA purification and mRNA determination by real-time PCR has been described before (Stiehl et al., 2006). Transcript levels of the HIF-dependent and -independent genes were quantified by reverse transcription (RT) quantitative (q) PCR using SybrGreen qPCR reagent kit (Sigma) in combination with an MX3000P light cycler (Stratagene). Initial copy number of each sample was calculated by comparison with serial dilutions of a calibrated standard. For mouse tissues, ribosomal protein S12 mRNA was used as a housekeeping gene while ribosomal protein L28 mRNA served as control for samples from human cell lines. Primer sequences are given in *Supplementary Table 1*.

Oxyblot detection of protein oxidation. PHD2 carbonylation was determined with a protein oxidation detection kit (Oxyblot, Millipore). Briefly, 50 ng/μl of recombinant PHD2 was exposed to either 100 μM $CoCl_2$, 10 μM $FeSO_4$, 2 mM

ascorbate, 0.5 mM 2-oxoglutarate or 1 mM H_2O_2 in the presence of 400 nM wild-type or P564A mutant HIF-1α peptide in 20 mM Tris-Cl, 5 mM KCl and 1.5 mM $MgCl_2$ for 1 hour at room temperature. Five μl of the reaction mix were mixed with 5 μl 12% SDS and carbonyl groups were derivatized with 10 mM 2,4-dinitrophenylhydrazine (DNPH) for 15 minutes. DNP groups were detected by immunoblotting using rabbit anti-DNP antibodies (1:150) followed by secondary goat anti-rabbit HRP-conjugated antibodies (1:300). For loading controls, PHD2 was detected using rabbit anti-PHD2 antibodies (Novus).

Animal studies. Gulo$^{-/-}$ mice were maintained on vitamin C–supplemented water containing 0.33 g/L of L-ascorbic acid and 0.01 mM EDTA as described earlier (Maeda et al., 2000). At 3 months of age, ascorbic acid supplement was withdrawn from six Gulo$^{-/-}$ males while continued for the control males. After 5 weeks, mice were killed with an overdose of 2,2,2-tribromoethanol, and tissues were collected and stored in RNAlater (Applied Biosystems Inc, Foster City, CA) at -20^{o}C until use. For hypoxia studies, 22 Gulo$^{-/-}$ males with an average age of 16 weeks were allocated to four groups so that no significant changes were observed in mean body weight and age of the animals at the beginning of the experiment. The body weight was determined every second day. After 36 days of ascorbate withdrawal, mice were exposed to 8% oxygen for 24 hours in a hypoxia tent (Coy Laboratory Products Inc.). Control animals were maintained at ambient oxygen concentration. Heparinized whole blood was collected from all mice by cardiac puncture after intraperitoneal injection anaesthesia using 4 mg/ml xylazine and 20 mg/ml ketamine at a dosage of 0.1 ml/20 g of body weight. Blood samples of hypoxic animals were collected inside the hypoxic tent. Animal experiments were conducted at two centers with the appropriate consent by the Institutional Animal Care and Use Committees of the University of North Carolina–Chapel Hill for breeding and normoxic gene expression studies or the Veterinary Office of the Canton Zürich (119/2010) for hypoxia studies, respectively.

Blood parameters and plasma EPO concentrations. Plasma EPO levels were measured by ELISA following the procedures recommended by the manufacturer (Quantikine, R&D Systems). EPO concentrations were determined by

comparison to a calibrated recombinant mouse EPO standard. Hematologic parameters of mouse whole blood were analyzed by the Division of Hematology (University Hospital, Zürich, Switzerland).

Results

Ascorbate is not required for HIF induction by hypoxia in HeLa cells.

HeLa cells have been described previously to grow in serum-free medium supplemented with hormones and growth factors (Hutchings and Sato, 1978). To achieve cell culture conditions avoiding ascorbate contamination derived from animal sera, Hela cells were adapted to a chemically defined medium free of ascorbate for at least 2 weeks. Control cells were grown in the same medium supplemented with 50 μM ascorbate. Both cell groups proliferated at the same rate with no differences noticed between ascorbate-free and supplemented cells (Fig. 1A). Despite its need for PHD activity *in vitro*, hypoxic HIF-1α protein accumulation was similar in ascorbate containing and deficient cells. However, a faint normoxic induction of HIF-1α could be observed in ascorbate-free cells only (Fig. 1B). Accordingly, cells transfected with a HIF-responsive reporter gene (pH3SVL) and subsequently exposed to graded oxygen concentrations (0.2, 1, 3 or 21% O_2, respectively) revealed similar induction levels of luciferase activity under both culture conditions (Fig. 1C).

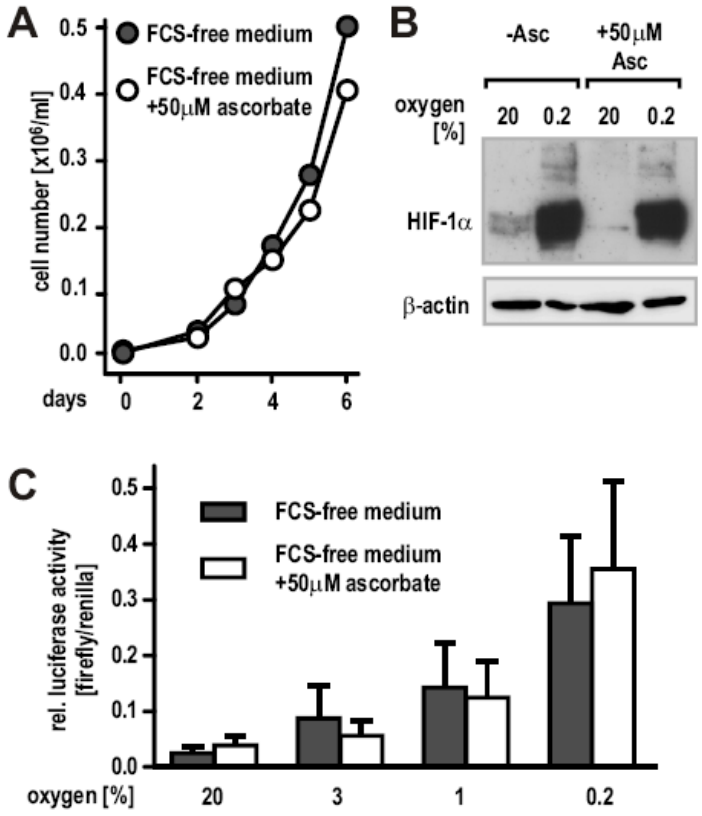

Fig 1. Cellular oxygen sensing by the PHD/HIF pathway does not require vitamin C. (A) Proliferation of HeLa cells growing in FCS-free, chemically defined medium containing either no or 50 μM ascorbate. (B) Stabilization of HIF-1α protein in HeLa cells maintained in FCS-free medium containing either no (-Asc.) or 50 μM ascorbate. Cells were exposed to 21 and 0.2% oxygen for 6 hours and protein levels analysed by immunoblotting. (C) Induction of HIF dependent luciferase activity (pH3SVL vector) in HeLa cells maintained in FCS-free medium containing either no or 50 μM ascorbate and exposed to 0.2%-21 % oxygen for 24 hours.

GSH can substitute for vitamin C in the hydroxylation reaction catalyzed by PHDs.

Because HeLa cells grown in a medium containing no ascorbate maintained hypoxic HIF-1α stabilization, we speculated that other antioxidants could compensate for vitamin C loss in these cells. Thus, a number of compounds with antioxidative properties were tested for their effects on PHD hydroxylation activity using a previously described *in vitro* hydroxylation assay (Wirthner et al., 2007). Surprisingly, some compounds such as n-propyl gallate and the superoxide dismutase mimetic Mn(III) tetrakis (1-methyl-4-pyridyl) porphyrin pentachloride (MnTMPyP) were potent inhibitors of PHD enzymes ((Nytko et al., 2007) and Supplementary Fig. 1A). However, reduced L-γ-glutamyl-L-cysteinyl-glycine (glutathione; GSH) enhanced HIFα hydroxylation by all three PHDs. Since the recombinant enzyme preparations in the initial experiments were expressed and purified as glutathione-S-transferase (GST) fusion proteins, we could not exclude interference of the tested antioxidants, particularly GSH, with the GST tag. Therefore, a PreScission protease cleavage site was engineered in between the two fusion partners. Chimeric GST.PHD and tag-free PHD enzymes showed comparable hydroxylation activity (Supplementary Fig. 1B). GSH increased the activity of all three untagged PHD isoforms, even in the complete absence of ascorbate (Fig. 2A). Addition of GSH ("+5 mM" in Fig. 2B) to 2 mM ascorbate increased the reaction rate when compared with ascorbate alone (“control” in Fig. 2B), suggesting two independent reaction modes of GSH, one replacing vitamin C and an additional one enhancing the reaction rate. Of note, only minor changes in ascorbate oxidation were found before and after one hour of PHD2-mediated substrate hydroxylation (Fig. 2C). The minor decrease in reduced ascorbate was most likely due to air-dependent oxidation rather than enzymatic consumption since it was independent of the presence of the hydroxyl-acceptor substrate (Fig. 2C, right panel). In conclusion, as shown previously for collagen P4H (Myllylä et al., 1984), ascorbate is not consumed during coupled PHD-catalyzed hydroxylation reactions.

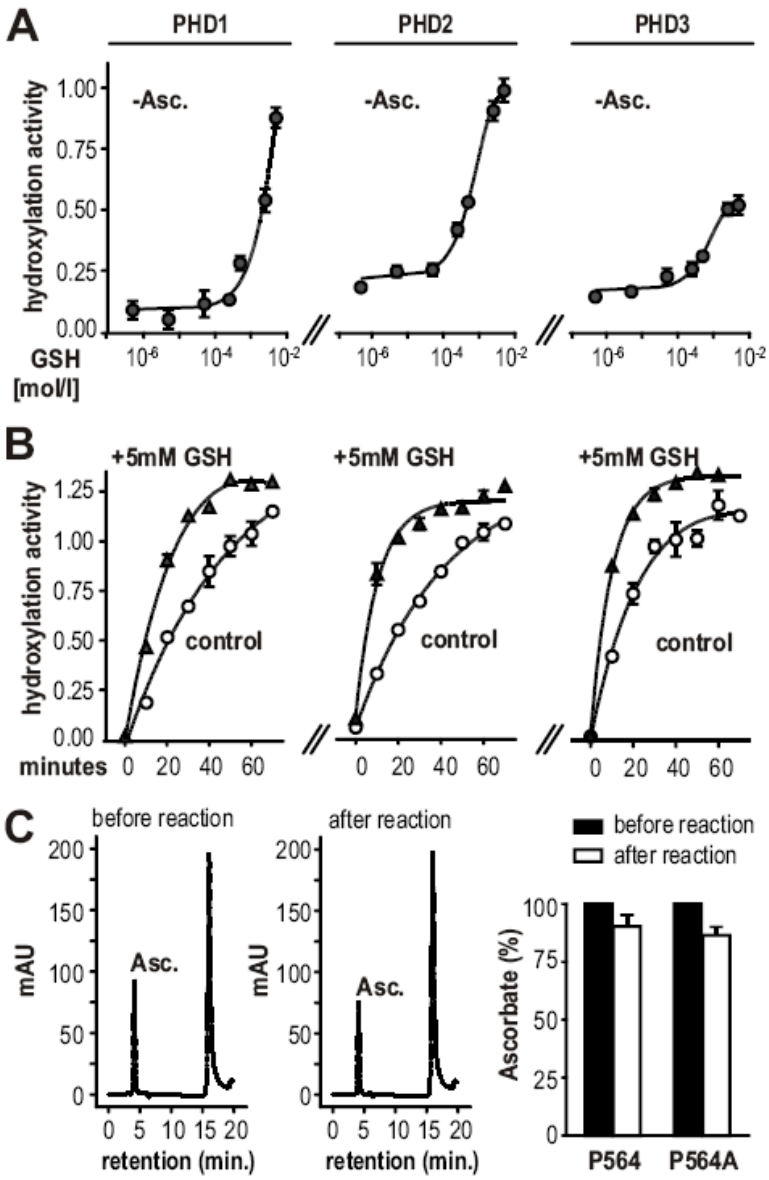

Fig. 2. GSH substitutes for vitamin C as a co-factor in HIF-α hydroxylation *in vitro*. (A) GSH can enhance PHD hydroxylation activity in the absence of ascorbate (-Asc.) in a dosedependent manner. Hydroxylation activity was determined using a multi-well VBC binding assay. (B) PHD-dependent hydroxylation reaction rate in the presence of 2 mM ascorbate (control) or 2 mM ascorbate combined with 5 mM GSH (+5 mM GSH). Shown are mean values ± SEM of triplicates. (C) Ascorbate determination by HPLC before and after 1 hour of PHD2-dependent hydroxylation reaction (left panels). Ascorbate content is only slightly decreased after 1 hour of incubation and independent of target hydroxylation (right panel). Shown are mean values ± SEM of three independent experiments normalized to values measured at time point zero.

GSH decreases HIF activity in $CoCl_2$-treated hepatoma cells.

In cell culture models, Co(II) and Ni(II) have been shown to substantially decrease cellular ascorbate content by catalyzing ascorbate oxidation to DHA followed by irreversible hydrolysis to diketogulonate (Salnikow and Kasprzak, 2005). Interestingly, exogenous ascorbate administration completely blunted the Co(II) induced hypoxic response in lung epithelial cells (Salnikow et al., 2004). To test if GSH could similarly compensate for reduced ascorbate levels after Co(II) stimulation, HIF transcriptional activity was further studied in HepG2 hepatoma cells stably transfected with a HIF-dependent luciferase reporter gene (HRG1 cells). We first determined the concentrations of $CoCl_2$ required to activate HIF-dependent reporter gene expression to a similar extent as exposure of the cells to 2% oxygen or the hypoxia mimicking iron chelator desferrioxamine mesylate (Dfx; Fig. 3A). Subsequently, HRG1 cells were treated with 100 μM $CoCl_2$ or 50 μM Dfx under 21% or 2% oxygen. Control cells were kept at ambient oxygen concentrations. All cells were co-treated with 1-10 mM GSH or 0.2-2 mM ascorbate. Indeed, ascorbate and GSH reduced HIF activity exclusively in $CoCl_2$-treated HRG1 cells (Fig. 3B). A substantial increase of HIF activation was noted particularly when cells were treated with 2 mM ascorbate, which might be explained by the pro-oxidative function ascorbate exerts if applied at high concentration (Fig. 3B, upper panel) (Chen et al., 2008). In line with these observations, both ascorbate and GSH reduced the expression levels of the endogenous HIF target genes CA9 and NDRG1 (Wenger et al., 2005) only in cells treated with $CoCl_2$, whereas 2 mM ascorbate enhanced hypoxic activation of both genes by almost 2-fold (Fig. 3C). To evaluate if these effects reflected differential activities of cellular PHD enzymes, HIF-1α protein accumulation was analyzed by immunoblotting. As expected, only $CoCl_2$-stabilized HIF-1α was down-regulated by co-treatment with ascorbate or GSH (Fig. 3D). Interestingly, 10 mM GSH was a more potent inhibitor of $CoCl_2$-induced HIF-1α stabilization than 2 mM ascorbate, a concentration which showed saturated inhibition of the HIF-reporter in the same cell line (Fig. 3B, upper panel)

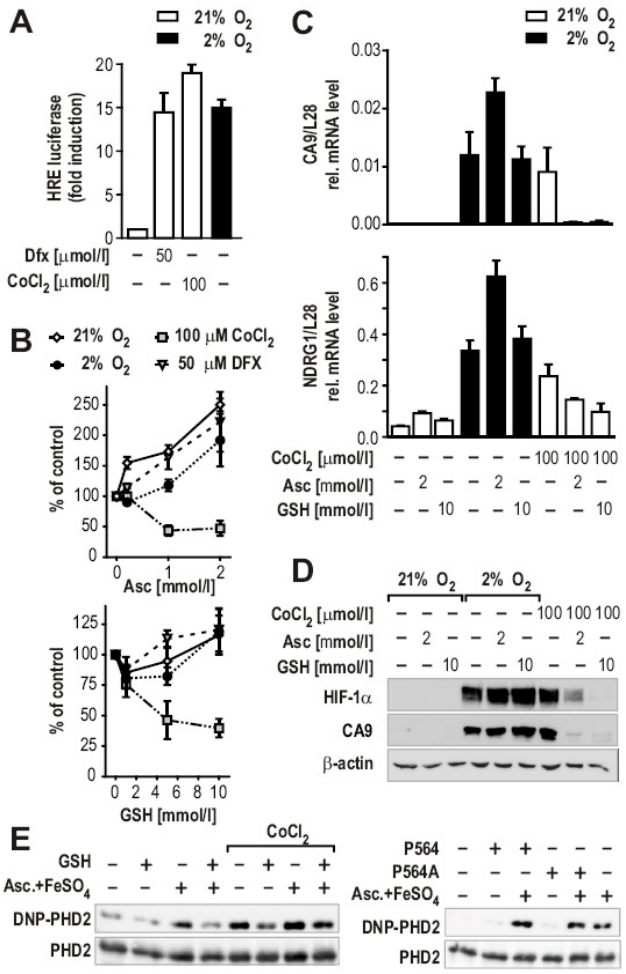

Fig. 3. GSH impairs HIF activation in cells. (A) Induction of HIF-dependent luciferase reporter gene activity in stably transfected HRG1 hepatoma cells by 2% O_2, 50 μM Dfx or 100 μM $CoCl_2$ for 24 hours. (B) Effects of ascorbate (Asc, 0.2-2 mM; upper panel) or GSH (1-10mM; lower panel) in combination with hypoxia, Dfx or $CoCl_2$ treatment on HIF-dependent luciferase activity relative to the protein concentration of the lysates. Shown are mean values ± SEM of three independent experiments normalized to the reporter activity in the absence of either ascorbate or GSH (control). (C) CA9 and NDRG1 HIF target gene mRNA levels in HRG1 cells following treatment with 2 mM ascorbate or 10 mM GSH combined with 2% O_2 or 100 μM $CoCl_2$ for 24 hours. Shown are mean values ± SEM of three independent experiments relative to the mRNA content of ribosomal protein L28. (D) HIF-1α and CA9 protein levels in HRG1 cellsfollowing treatment with 2 mM ascorbate or 10 mM GSH combined with 2% O_2 or 100 μM $CoCl_2$. (E) Oxyblot analyses of recombinant PHD2 protein carbonylation. GSH (5 mM) reduced PHD2 carbonylation by either 2 mM ascorbate/10 μM $FeSO_4$ or 100 μM $CoCl_2$ (left panel). PHD2 oxidation is independent of target hydroxylation as shown by using a wild-type or a P564A mutant HIF-1α hydroxyl-proline acceptor peptide in *in vitro* hydroxylation reactions (right panel).

GSH protects PHD2 from metal-catalyzed oxidation.

Enzymatic activity of the PHDs is sensitive to reactive oxygen species (ROS) and transition metal ions (Acker et al., 2006). However, the mechanism(s) by which ROS or metal ions inhibit hydroxylase activity remained speculative. Besides its general antioxidative properties as radical scavenger, vitamin C actively interferes with the oxidation state of metal ions by serving as electron donor in a redox reaction. As such it largely differs from GSH which is a major cellular antioxidant protecting cysteinyl and methionyl residues in proteins from oxidative modifications. Both, $CoCl_2$ and H_2O_2 inhibited all three PHD isoforms *in vitro* with PHD2 being slightly more resistant to $CoCl_2$ (Supplementary Fig. 3). To directly determine protein oxidation by these compounds, carbonyl group formation in PHD2 was estimated by Oxyblot technology. As shown in Fig. 3E (left panel), 2 mM ascorbate and 10 μM $FeSO_4$ (as used in the standard reaction buffer for *in vitro* hydroxylation) substantially increased carbonylation of recombinant PHD2 during one hour of hydroxylation reaction. Surprisingly, $CoCl_2$ increased carbonylation of PHD2 whether ascorbate/$FeSO_4$ was present or not. Following addition of 5 mM GSH, oxidation of PHD2 by ascorbate/$FeSO_4$ and $CoCl_2$ was markedly reduced. To examine whether PHD2 protein oxidation is coupled to its di-oxygenase activity, the reaction was performed in the presence of a mutant P564A HIF-1α peptide substrate. As shown in Fig. 3E (right panel), PHD2 protein oxidation was independent of the presence of a hydroxylation acceptor proline, providing evidence that protein oxidation is not caused by the hydroxylation reaction cycle.

Cysteine 201 affects PHD2 hydroxylation activity.

Recently, cysteine residue C201 within the catalytic domain of PHD2 has been identified as a surface accessible, highly nucleophilic residue predominantly interacting with thiol compounds (Mecinovic et al., 2009). Moreover, C201 and C208 were proposed to provide an additional metal binding site in PHD2 (Mecinovic et al., 2008). Both, C201 and C208 are highly conserved among all three human and mouse PHD isoforms (Fig. 4A). To investigate the functional relevance of C201, recombinant PHD2, wild-type or C201S mutant, was purified from Sf9 cells. Surprisingly, the C201S mutation significantly ($p<0.0001$) increased the PHD2 reaction rate by 2.5-fold (Fig. 4B, upper panel). Equal concentrations of wild-type and mutant PHD2 proteins were confirmed by Coomassie staining of the undiluted stock solutions and immunoblotting of the diluted assay solutions (Fig. 4B, lower panel). To further test the hypothesis that the C201S mutation might protect the PHD2 enzyme from oxidative damage, the effect of H_2O_2 on hydroxylation activity was measured. As shown in Fig. 4C, the half-maximal inhibitory concentration (IC_{50}) for H_2O_2 was roughly 5-fold higher for the C201S mutant compared with wild-type PHD2 (IC_{50} of 8.9×10^{-6} and 4.3×10^{-5} M H_2O_2 for wild-type and C201S mutant PHD2, respectively). We further tested if the H_2O_2 mediated loss of PHD2 activity could be rescued by sequential addition of GSH. For both enzyme preparations, pre-incubation with 1 mM H_2O_2 for 30 minutes inhibited subsequent substrate hydroxylation reactions, inspite of 2 mM ascorbate being freshly added to start the hydroxylation reaction (Fig 4D). Interestingly, further addition of 5 mM GSH could similarly reactivate both enzyme preparations, though reaction kinetics were substantially slower for the reactivated enzymes. (Fig. 4D).

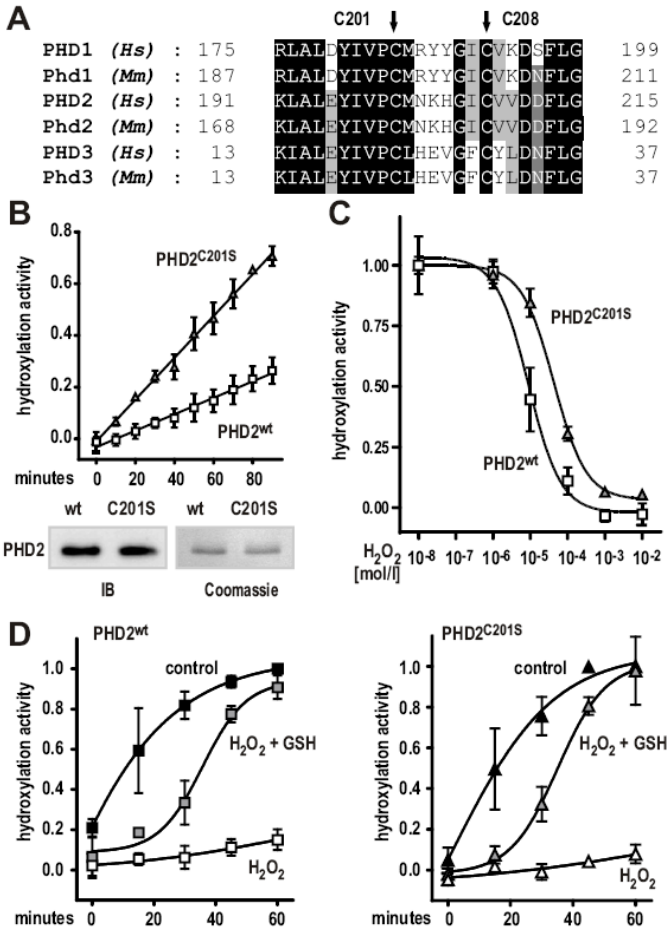

Fig. 4. A C201S mutation enhances PHD2-dependent hydroxylation reaction rate and protects from protein oxidation. (A) Conservation of cysteine residues (C201 and C208 in human PHD2) in all three human (*Hs*) and mouse (*Mm*) PHD isoforms. (B) Increased reaction rate of C201S mutant PHD2 as measured by the hydroxylation-dependent VBC binding assay. Shown are mean values ± SEM of three independent experiments. Linear regression analyses were performed, revealing highly different slopes ($p<0.0001$). (C) The C201S mutation confers resistance of PHD2 to H_2O_2-mediated inhibition of hydroxylation activity. Shown are mean values ± SEM of a representative experiment performed in triplicates. (D) Reduced glutathione (GSH) can rescue PHD2 wt and C201S hydroxylation activities after H_2O_2 -mediated enzyme damage. Briefly, enzyme preparations were pre-incubated with 1 mM H_2O_2 for 30 minutes(H_2O_2) or left untreated for a similar period (control). For rescue experiments, enzymes after H_2O_2 treatment were incubated with 5 mM GSH (H_2O_2+GSH) for 15 minutes. Hydroxylation reactions were carried out at standard assay conditions for 60 minutes. Note that all reactions contained 2 mM ascorbate freshly added when hydroxylation reactions were started. Data is given as mean values ± SEM of three independent experiments normalized to hydroxylation activities of control reactions obtained after 60 minutes.

Oxygen sensing is fully functional in ascorbate-deficient Gulo$^{-/-}$ mice.

Gulo$^{-/-}$ mice received a diet with or without vitamin C for 5 weeks. Transcript levels of known HIF target genes (Bnip3, Ca9, Glut1, Pdk1, Phd2, Phd3, Ndrg1) as well as genes involved in antioxidative defense (Sod1, Sod2, Glrx), the ascorbate transporters Svct1 and Svct2 or oxygen-independent genes (Ednrb, Mmp3, Phd1) were determined by RT-qPCR in brain, lung, kidney, heart and liver. Similar tissue-specific expression levels for most genes involved in different pathways were observed in both groups (Fig. 5A). Conclusive with our finding from cell culture experiments, expression levels of most of the HIF target genes remained largely unaffected by vitamin C deficiency or even showed reduced levels (blank and blue squares, respectively, in the heatmap shown in Fig. 5B). Expression of the ascorbate transporter Svct1, however, was moderately induced in animals fed without ascorbate, possibly reflecting compensatory mechanisms for ascorbate deprivation.

To further test if the absence of ascorbate limits PHD function under hypoxic conditions, Gulo$^{-/-}$ males were deprived from vitamin C for 5 weeks, while control animals were supplemented with vitamin C. As described previously (Maeda et al., 2000), the animals developed a scorbutic phenotype marked by a substantial loss of body weight after 35 days on a ascorbate-free diet, indicating that systemic stores of vitamin C have been exhausted (Fig. 5C, left panel). In line with this observation, plasma ascorbate levels in Gulo$^{-/-}$ mice fed an ascorbate-free diet were below the detection limit (≤1 μM), while plasma of control animals contained 40.3-123.8 μM of vitamin C (Fig. 5C, right panel), corresponding to ascorbate plasma levels in healthy humans (Levine et al., 1996).

Following 5 weeks of vitamin C deprivation, mice were breathing 8% oxygen for an additional period of 24 hours while control groups were kept under ambient oxygen concentration (see scheme in Fig. 5C). Both ascorbate-deficient and supplemented animals responded to the hypoxic treatment with a robust induction of *Epo* mRNA in the kidney which was higher in Gulo$^{-/-}$ males fed without ascorbate, though differences did not reach the level of significance ($p = 0.07$, Student's t-test) (Fig. 5D, left panel). Circulating EPO protein levels in mouse plasma were induced by hypoxic exposure to a similar extent in both groups (Fig. 5C, right panel). Of note, no significant changes of the red cell lineage hematologic parameters were observed in

Gulo$^{-/-}$ mice after 5 weeks of ascorbate depletion, indicating that the oxygen transport capacity was similar in both treatment groups. Hypoxic increases in hematocrit values are known to be delayed and reach the level of significance not before 72 hours of continuous exposure to hypoxia (Seferynska et al., 1989), explaining the lack of an increase of either hematocrit values or red blood cell counts in our experimental setting with a hypoxic period of only 24 hours (Supplementary Table 2).

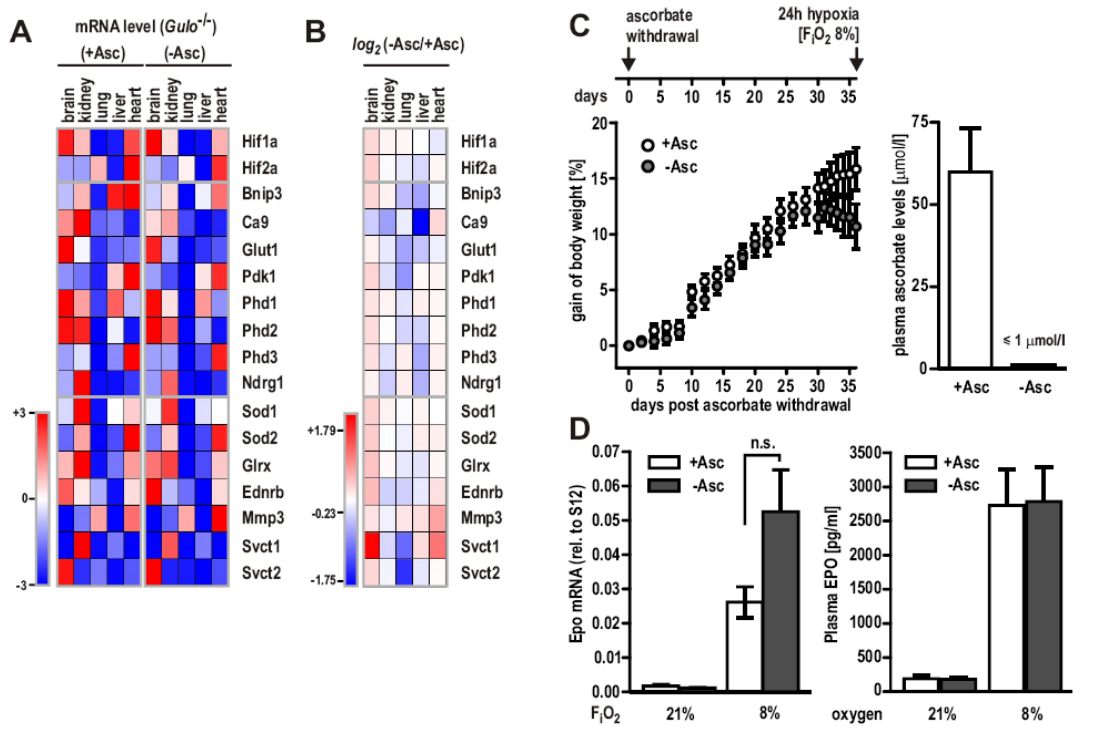

Fig. 5. The hypoxic response is fully functional in vitamin C depleted Gulo$^{-/-}$ **mice.** Gulo$^{-/-}$ male mice received a diet with (+Asc) or without (-Asc) ascorbate for five weeks. (A) Transcript levels of HIF target genes (Bnip3, Ca9, Glut1, Pdk1, Phd2, Phd2, Phd3, Ndrg1) as well as genes involved in antioxidative defense (Sod1, Sod2, Glrx), the ascorbate transporters Svct1 and Svct2 or oxygen-independent genes (Ednrb, Mmp3, Phd1) were quantified by RT-qPCR in brain, kidney, lung, liver and heart. Values are expressed relative to S12 mRNA levels and visualized in a heat map (Genepattern, Broad Institute, USA). Lowest and highest mRNA levels of each gene were arbitrarily defined as -3 (dark blue) and +3 (dark red), respectively. (B) Heat map of gene expression changes following a vitamin-C-deficient diet. Log2 (-Asc/+Asc) ratios revealed that the majority of HIF target genes remained either unchanged or showed a slightly reduced expression pattern. Data ranged from -1.75 (dark blue) and +1.79 (dark red), respectively. (C) Scheme depicting the experimental setup for hypoxic experiments with vitamin C depleted Gulo$^{-/-}$ animals (upper panel). Relative gain of body weight of Gulo$^{-/-}$ mice (n=11 animals per group) following ascorbate withdrawal (-Asc) or ascorbate supplementation (+Asc) for 36 days (left panel). Ascorbate levels in the plasma of Gulo$^{-/-}$ mice (n=6 animals per group) after five weeks of ascorbate withdrawal compared to mice kept on a ascorbate-supplemented diet (right panel). (D) Epo mRNA (left panel) and circulating EPO protein (right panel) levels in Gulo$^{-/-}$ mice maintained on a diet with (+Asc) or without (-Asc) for five weeks followed by exposure to 8% or 21% oxygen for 24 hours. Data represent mean values ± SEM derived from at least 5 animals per group (n.s., not significant).

Discussion

Ascorbic acid and ferrous iron have been reported as essential co-factors for PHD-dependent HIFα hydroxylation *in vitro* (Hirsilä et al., 2003). Unexpectedly, we found a fully functional cellular oxygen sensing pathway in HeLa cells maintained under strictly ascorbate-free culture conditions, indicating that ascorbate is dispensable for HIFα hydroxylation *in vivo*. In search for the nature of antioxidative compounds substituting for ascorbate during prolyl-4-hydroxylation, we identified GSH as a potent activator of all three PHDs *in vitro,* increasing HIFα peptide hydroxylation in a dose-dependent manner. Notably, ascorbate and reduced glutathione are the most abundant reducing compounds within eukaryotic cells (Linster and Van Schaftingen, 2007). Given the distinct antioxidative properties of vitamin C and GSH, the two compounds might affect prolyl-4-hydroxylation by different ways. Ascorbate might be required to reduce occasionally oxidized ferric Fe(III) generated in the active centre of PHDs by uncoupled reaction cycles as it has been described for collagen P4H (Myllylä et al., 1984). However, to the best of our knowledge, no experimental evidence has been reported for enzymatic activity of PHDs in the absence of a hydroxyl-acceptor substrate. Strikingly, the major iron form bound to purified PHD2 is ferrous Fe(II) even when purified under oxygenated conditions (McNeill et al., 2005), arguing against an essential role of ascorbate in reducing PHD iron. In support of this notion, iron and 2-oxoglutarate have been reported to co-purify with 50% and 5-10% of PHD2, respectively, while ascorbate did not co-purify at all (McNeill et al., 2005). Recent work by Flashman *et al.* showed that ascorbate does not directly interact with the catalytic domain of PHD2, however, its intrinsic ene-diol reducing moiety was found to be important to promote hydroxylation by PHD2 (Flashman et al., 2010).

GSH fully stimulated *in vitro* PHD hydroxylation activity only at rather high concentrations, which is in line with findings previously reported for N-terminally truncated PHD2 (Flashman et al.). The millimolar GSH concentrations used in our study reflect physiologically relevant levels of this compound like they occur in living cells (Meister and Anderson, 1983). Moreover, we found that ascorbic acid is not consumed by coupled substrate hydroxylation, suggesting that exogenously added GSH does not simply regenerate potentially co-purified oxidized dehydroascorbate

(DHA). Our data rather favor an alternative function of GSH by preventing oxidative damage to the enzyme itself. Physiological concentrations of GSH were able to reduce transition metal- or peroxide-induced PHD enzyme carbonylation. Despite being generally referred to as antioxidant, ascorbate, together with oxygen and transition metals such as Fe(III) or Cu(II), also exerts pro-oxidative effects by generating hydroxyl radicals in a Fenton-like reaction (Stich et al., 1976). Indeed, we found increased PHD2 carbonylation by ascorbate/iron *in vitro*, suggesting that GSH might protect PHDs from the adverse effects of ascorbate. In line with two distinct reaction modes, addition of GSH to hydroxylation reactions containing saturating ascorbate concentrations markedly increased the hydroxylation rate of PHDs *in vitro*. Of note, Co(II) induced ascorbate depletion, as suggested for cultured cells (Salnikow et al., 2004; Salnikow and Kasprzak, 2005), cannot account for PHD inhibition in our cell-free assays, since we previously showed that Co(II) only inefficiently catalyses ascorbate oxidation by air under these assay conditions (Nytko et al., 2007). Direct interference of Co(II) with the enzymes is supported by the observation that Co(II) strongly carbonylated purified PHD2 even in the complete absence of ascorbate. Moreover, ascorbate and GSH exclusively blunted Co(II)-induced HIF activation in our cellular models, demonstrating a complementary function of GSH and ascorbate in oxygen sensing by living cells. While simple chelation of Co(II) by ascorbate and GSH cannot be fully excluded in cell culture experiments, it should be mentioned that metal chelators naturally occurring in serum (e.g. histidine, glutamic acid, albumin but also GSH) are essential to facilitate Co(II) induced ascorbate oxidation, since “free” Co(II) is unable to directly oxidize ascorbate in simple aqueous solutions at neutral pH (reviewed in (Salnikow and Kasprzak, 2005). Thus, the actual redox potential of the ion in such ternery complexes - rather than the intracellular concentration of “free” metal - determines its efficacy to act as a “hypoxia mimetic”.

One of the specific GSH functions is to prevent or reduce inappropriate disulfide bond formation. Two recent reports identified PHD2 cysteinyl residues C201 and C208 to be highly nucleophilic and surface accessible (Mecinovic et al., 2009; Mecinovic et al., 2008). Moreover, crystallographic analyses predicted that these two cysteines might form disulfide bonds (Mecinovic et al., 2009; Mecinovic et al., 2008). C201S mutation protects recombinant PHD2 from oxidative damage *in vitro* and results in a 2.5-fold higher specific hydroxylation activity. One might speculate that a

certain fraction of wild-type PHD2 enzyme constantly undergoes oxidative modification of C201, leading to reduced activity. As such, PHD enzymes could combine oxygen and redox sensing properties, providing a possible explanation for previous work on redox factors modulating PHD activity (Gerald et al., 2004; Lu et al., 2005; Pan et al., 2007). However, potentiation of PHD activity by GSH clearly involves mechanisms distinct from C201 oxidation, since both, wildtype and C201S mutated PHD2 enzymes could be efficiently reactivated from H_2O_2-induced damage by GSH.

Translating our biochemical and cellular findings in a systemic context, we did not observe marked alterations in HIF target gene expression following dietary vitamin C deprivation in Gulo$^{-/-}$ mice, providing evidence that other antioxidants might substitute for vitamin C *in vivo*. Accordingly, the hypoxic response of Gulo$^{-/-}$ mice with low or undetectable ascorbate in the plasma was similar to mice receiving an ascorbate-supplemented diet. Consistent with antioxidant redundancy *in vivo*, a study using the same animals backcrossed to a BALB/c genetic background found vitamin C-independent *de novo* synthesis of collagen in allografted tumors and unchanged levels of hydroxyproline-collagen. Dermal hydroxyproline content of collagen was even increased (Parsons et al., 2006). Of note, treatment of vitamin C-deprived guinea pigs with a cell permeable GSH monoethyl ester significantly attenuated the severity of scurvy-related symptoms, suggesting a cooperative function of both antioxidants for P4H function also *in vivo* (Martensson et al., 1993). Moreover, Gulo$^{-/-}$ mice have increased levels of total glutathione in brain and liver, possibly explained by a compensatory mechanism for antioxidative defense in these animals (Harrison et al., 2010).

Interestingly, tumor growth and angiogenesis was retarded in a syngenic tumor model using vitamin C-deprived Gulo$^{-/-}$ mice, but no changes were observed for HIF-1α protein levels in the respective tumor tissue (Telang et al., 2007). The possible value of vitamin C in cancer therapy recently experienced a renaissance, since it has been shown that pharmacological doses of vitamin C decrease growth of tumor xenografts in mice by increasing peroxide levels in neoplastic tissue (Chen et al., 2005; Chen et al., 2008). Such tumoricidal effects of antioxidants might, at least partially, involve destabilization of HIFα following increased hydroxylase activity (Gao et al., 2007; Knowles et al., 2003). Our data support a model of cooperative

function of GSH and vitamin C in regulating the efficiency of PHD oxygen sensors. Translated to chemotherapy of cancers, combined treatment with both clinically approved molecules might even boost their antitumorigenic function.

Acknowledgments

We would like to thank F. Oehme, S. Pastorekova and D. Peet for providing materials and B. Blattmann and M. Bordoli for technical advise. This work was supported by NIH grant HL42630 (to N.M.), the Swiss National Science Foundation grant 31003A_129962/1 (to R.H.W. and D.P.S.) and the *Forschungskredit* of the University of Zurich (to D.P.S.).

Autorship Contributions

K.J.N. designed and performed experiments, analyzed data, and wrote the manuscript. K.J.N., N.M., Ph.S. and Pa.S. performed *in vivo* experiments. R.H.W designed experiments and wrote the manuscript. D.P.S designed experiments, analyzed data, and wrote the manuscript.

Conflict of Interest Disclosures

The authors declare no competing financial interests.

References

Acker T, Fandrey J, Acker H (2006). The good, the bad and the ugly in oxygen-sensing: ROS, cytochromes and prolyl-hydroxylases. Cardiovasc Res 71(2): 195-207.

Berra E, Benizri E, Ginouvés A, Volmat V, Roux D, Pouysségur J (2003). HIF prolyl-hydroxylase 2 is the key oxygen sensor setting low steady-state levels of HIF-1α in normoxia. EMBO J 22(16): 4082-90.

Bruick RK, McKnight SL (2001). A conserved family of prolyl-4-hydroxylases that modify HIF. Science 294(5545): 1337-40.

Chen Q, Espey MG, Krishna MC, Mitchell JB, Corpe CP, Buettner GR, Shacter E, Levine M (2005). Pharmacologic ascorbic acid concentrations selectively kill cancer cells: action as a pro-drug to deliver hydrogen peroxide to tissues. Proc Natl Acad Sci USA 102(38): 13604-9.

Chen Q, Espey MG, Sun AY, Pooput C, Kirk KL, Krishna MC, Khosh DB, Drisko J, Levine M (2008). Pharmacologic doses of ascorbate act as a prooxidant and decrease growth of aggressive tumor xenografts in mice. Proc Natl Acad Sci USA 105(32): 11105-9.

Chowdhury R, McDonough MA, Mecinovic J, Loenarz C, Flashman E, Hewitson KS, Domene C, Schofield CJ (2009). Structural basis for binding of hypoxia-inducible factor to the oxygen-sensing prolyl hydroxylases. Structure 17(7): 981-9.

Flashman E, Davies SL, Yeoh KK, Schofield CJ (2010). Investigating the dependence of the hypoxia-inducible factor hydroxylases (factor inhibiting HIF and prolyl hydroxylase domain 2) on ascorbate and other reducing agents. Biochem 427(1): 135-42.

Flashman E, Hoffart LM, Hamed RB, Bollinger Jr JM, Krebs C, Schofield CJ (2010). Evidence for the slow reaction of hypoxia-inducible factor prolyl hydroxylase 2 with oxygen. Febs J 2010: 31.

Gao P, Zhang H, Dinavahi R, Li F, Xiang Y, Raman V, Bhujwalla ZM, Felsher DW, Cheng L, Pevsner J, Lee LA, Semenza GL, Dang CV (2007). HIF-dependent antitumorigenic effect of antioxidants in vivo. Cancer Cell 12(3): 230-8.

Gerald D, Berra E, Frapart YM, Chan DA, Giaccia AJ, Mansuy D, Pouysségur J, Yaniv M, Mechta-Grigoriou F (2004). JunD reduces tumor angiogenesis by protecting cells from oxidative stress. Cell. 118(6): 781-94.

Harrison FE, Meredith ME, Dawes SM, Saskowski JL, May JM (2010). Low ascorbic acid and increased oxidative stress in gulo(-/-) mice during development. Brain Res (19): 143-52.

Hewitson KS, McNeill LA, Riordan MV, Tian YM, Bullock AN, Welford RW, Elkins JM, Oldham NJ, Bhattacharya S, Gleadle JM, Ratcliffe PJ, Pugh CW, Schofield CJ (2002). Hypoxia-inducible factor (HIF) asparagine hydroxylase is identical to factor inhibiting HIF (FIH) and is related to the cupin structural family. J Biol Chem. 277(29): 26351-5.

Hirsilä M, Koivunen P, Günzler V, Kivirikko KI, Myllyharju J (2003). Characterization of the human prolyl 4-hydroxylases that modify the hypoxia-inducible factor. J Biol Chem 278(33): 30772-80.

Hutchings SE, Sato GH (1978). Growth and maintenance of HeLa cells in serum-free medium supplemented with hormones. Proc Natl Acad Sci U S A. 75(2): 901-4.

Ivan M, Kondo K, Yang H, Kim W, Valiando J, Ohh M, Salic A, Asara JM, Lane WS, Kaelin WG, Jr. (2001). HIFα targeted for VHL-mediated destruction by proline hydroxylation: implications for O2 sensing. Science 292(5516): 464-8.

Jaakkola P, Mole DR, Tian YM, Wilson MI, Gielbert J, Gaskell SJ, Kriegsheim A, Hebestreit HF, Mukherji M, Schofield CJ, Maxwell PH, Pugh CW, Ratcliffe PJ (2001). Targeting of HIF-α to the von Hippel-Lindau ubiquitylation complex by O2-regulated prolyl hydroxylation. Science 292(5516): 468-72.

Kaelin WG, Jr., Ratcliffe PJ (2008). Oxygen sensing by metazoans: the central role of the HIF hydroxylase pathway. Mol Cell. 30(4): 393-402.

Knowles HJ, Raval RR, Harris AL, Ratcliffe PJ (2003). Effect of ascorbate on the activity of hypoxia-inducible factor in cancer cells. Cancer Res 63(8): 1764-8.

Ladroue C, Carcenac R, Leporrier M, Gad S, Le Hello C, Galateau-Salle F, Feunteun J, Pouysségur J, Richard S, Gardie B (2008). PHD2 mutation and congenital erythrocytosis with paraganglioma. N Engl J Med 359(25): 2685-92.

Levine M, Conry-Cantilena C, Wang Y, Welch RW, Washko PW, Dhariwal KR, Park JB, Lazarev A, Graumlich JF, King J, Cantilena LR (1996). Vitamin C pharmacokinetics in healthy volunteers: evidence for a recommended dietary allowance. Proc Natl Acad Sci U S A. 93(8): 3704-9.

Linster CL, Van Schaftingen E (2007). Vitamin C. Biosynthesis, recycling and degradation in mammals. Febs J. 274(1): 1-22.

Lu H, Dalgard CL, Mohyeldin A, McFate T, Tait AS, Verma A (2005). Reversible inactivation of HIF-1 prolyl hydroxylases allows cell metabolism to control basal HIF-1. J Biol Chem 280(51): 41928-39.

Maeda N, Hagihara H, Nakata Y, Hiller S, Wilder J, Reddick R (2000). Aortic wall damage in mice unable to synthesize ascorbic acid. Proc Natl Acad Sci USA 97(2): 841-6.

Mandl J, Szarka A, Banhegyi G (2009). Vitamin C: update on physiology and pharmacology. Br J Pharmacol. 157(7): 1097-110.

Martensson J, Han J, Griffith OW, Meister A (1993). Glutathione ester delays the onset of scurvy in ascorbate-deficient guinea pigs. Proc Natl Acad Sci USA 90(1): 317-21.

McNeill LA, Flashman E, Buck MR, Hewitson KS, Clifton IJ, Jeschke G, Claridge TD, Ehrismann D, Oldham NJ, Schofield CJ (2005). Hypoxia-inducible factor prolyl hydroxylase 2 has a high affinity for ferrous iron and 2-oxoglutarate. Mol Biosyst 1(4): 321-4.

Mecinovic J, Chowdhury R, Flashman E, Schofield CJ (2009). Use of mass spectrometry to probe the nucleophilicity of cysteinyl residues of prolyl hydroxylase domain 2. Anal Biochem 393(2): 215-21.

Mecinovic J, Chowdhury R, Liénard BM, Flashman E, Buck MR, Oldham NJ, Schofield CJ (2008). ESI-MS studies on prolyl hydroxylase domain 2 reveal a new metal binding site. Chem Med Chem 3(4): 569-72.

Meister A, Anderson ME (1983). Glutathione. Annu Rev Biochem. 52: 711-60.

Mody VC, Jr., Kakar M, Elfving A, Soderberg PG, Lofgren S (2005). Ascorbate in the rat lens: dependence on dietary intake. Ophthalmic Res 37(3): 142-9.

Myllylä R, Majamaa K, Günzler V, Hanauske-Abel HM, Kivirikko KI (1984). Ascorbate is consumed stoichiometrically in the uncoupled reactions catalyzed by prolyl 4-hydroxylase and lysyl hydroxylase. J Biol Chem 259(9): 5403-5.

Nytko KJ, Spielmann P, Camenisch G, Wenger RH, Stiehl DP (2007). Regulated function of the prolyl-4-hydroxylase domain (PHD) oxygen sensor proteins. Antioxid Redox Signal 9(9): 1329-38.

Pan Y, Mansfield KD, Bertozzi CC, Rudenko V, Chan DA, Giaccia AJ, Simon MC (2007). Multiple factors affecting cellular redox status and energy metabolism modulate hypoxia-inducible factor prolyl hydroxylase activity in vivo and in vitro. Mol Cell Biol 27(3): 912-25.

Parsons KK, Maeda N, Yamauchi M, Banes AJ, Koller BH (2006). Ascorbic acid-independent synthesis of collagen in mice. Am J Physiol Endocrinol Metab 290(6): E1131-9.

Percy MJ, Furlow PW, Beer PA, Lappin TR, McMullin MF, Lee FS (2007). A novel erythrocytosis-associated PHD2 mutation suggests the location of a HIF binding groove. Blood 110(6): 2193-6.

Percy MJ, Zhao Q, Flores A, Harrison C, Lappin TR, Maxwell PH, McMullin MF, Lee FS (2006). A family with erythrocytosis establishes a role for prolyl hydroxylase domain protein 2 in oxygen homeostasis. Proc Natl Acad Sci USA 103(3): 654-9.

Salnikow K, Donald SP, Bruick RK, Zhitkovich A, Phang JM, Kasprzak KS (2004). Depletion of intracellular ascorbate by the carcinogenic metals nickel and cobalt results in the induction of hypoxic stress. J Biol Chem 279(39): 40337-44.

Salnikow K, Kasprzak KS (2005). Ascorbate depletion: a critical step in nickel carcinogenesis? Environ Health Perspect 113(5): 577-84.

Seferynska I, Brookins J, Rice JC, Fisher JW (1989). Erythropoietin production in exhypoxic polycythemic mice. Am J Physiol. 256(4 Pt 1): C925-9.

Simoes SI, Eleuterio CV, Cruz ME, Corvo ML, Martins MB (2003). Biochemical changes in arthritic rats: dehydroascorbic and ascorbic acid levels. Eur J Pharm Sci 18(2): 185-9.

Stich HF, Karim J, Koropatnick J, Lo L (1976). Mutogenic action of ascorbic acid. Nature 260(5553): 722-4.

Stiehl DP, Jelkmann W, Wenger RH, Hellwig-Burgel T (2002). Normoxic induction of the hypoxia-inducible factor 1alpha by insulin and interleukin-1beta involves the phosphatidylinositol 3-kinase pathway. FEBS Lett 512(1-3): 157-62.

Stiehl DP, Wirthner R, Köditz J, Spielmann P, Camenisch G, Wenger RH (2006). Increased prolyl 4-hydroxylase domain proteins compensate for decreased oxygen levels. Evidence for an autoregulatory oxygen-sensing system. J Biol Chem 281(33): 23482-91.

Takeda K, Aguila HL, Parikh NS, Li X, Lamothe K, Duan LJ, Takeda H, Lee FS, Fong GH (2008). Regulation of adult erythropoiesis by prolyl hydroxylase domain proteins. Blood 111(6): 3229-35.

Takeda K, Cowan A, Fong GH (2007). Essential role for prolyl hydroxylase domain protein 2 in oxygen homeostasis of the adult vascular system. Circulation 116(7): 774-81.

Takeda K, Ho VC, Takeda H, Duan LJ, Nagy A, Fong GH (2006). Placental but not heart defects are associated with elevated hypoxia-inducible factor α levels in mice lacking prolyl hydroxylase domain protein 2. Mol Cell Biol 26(22): 8336-46.

Telang S, Clem AL, Eaton JW, Chesney J (2007). Depletion of ascorbic acid restricts angiogenesis and retards tumor growth in a mouse model. Neoplasia 9(1): 47-56.

Wanner RM, Spielmann P, Stroka DM, Camenisch G, Camenisch I, Scheid A, Houck DR, Bauer C, Gassmann M, Wenger RH (2000). Epolones induce erythropoietin expression via hypoxia-inducible factor-1 alpha activation. Blood. 96(4): 1558-65.

Wenger RH, Stiehl DP, Camenisch G (2005). Integration of oxygen signaling at the consensus HRE. Sci STKE 2005(306): re12.

Wirthner R, Balamurugan K, Stiehl DP, Barth S, Spielmann P, Oehme F, Flamme I, Katschinski DM, Wenger RH, Camenisch G (2007). Determination and modulation of prolyl-4-hydroxylase domain oxygen sensor activity. Methods Enzymol 435: 43-60.

Author Manuscript II published in Blood, 2011 Feb 23. [Epub ahead of print].
PMID: 21346252

Supplementary material to Nytko *et al.,* " Vitamin C is dispensable for oxygen sensing *in vivo*"

Inventory of supplemental information

Supplementary Table 1 relates to Material and Methods. Detailed primer information for quantitative PCR analyses.

Supplementary Table 2 relates to Figure 5. Heamatologic blood parameters of $Gulo^{-/-}$ mice kept on a vitamin C free diet.

Supplementary Figure 1 relates to Figure 2. Generation and *in vitro* activity of untagged PHD enzymes.

Supplementary Figure 2 relates to Figure 2. Determination of ascorbate concentration in hydroxylation reactions by HPLC.

Supplementary Figure 3 relates to Figure 3. $CoCl_2$ and H_2O_2 inhibit *in vitro* hydroxylation activity of PHD1-3.

Author Manuscript II published in Blood, 2011 Feb 23. [Epub ahead of print].
PMID: 21346252

Table S1. **Sequence of human and mouse primers used for RT-qPCR measurements.**
Ca9, carbonic anhydrase IX; Ndrg1, N-myc downstream regulated gene 1; L28, ribosomal protein L28; Hif1a, hypoxia inducible factor 1, α subunit; Hif2a, hypoxia inducible factor 2, α subunit; Bnip3, BCL2/adenovirus E1B interacting protein 1; Glut1, glucose transporter, member 1; Pdk1, pyruvate dehydrogenase kinase, isoenzyme 1; Phd1-3, prolyl-4-hydroxylase domain 1-3; Sod1, superoxide dismutase 1; Sod2, superoxide dismutase 2; Glrx, glutaredoxin; Ednrb, endothelin receptor type B; Mmp3, matrix metallopeptidase 3; Svct1, sodium-dependent vitamin C transporter 1; Svct2, sodium-dependent vitamin C transporter 2; Epo, erythropoietin; S12, Ribosomal protein S12.

Gene	Species	Accession No.	Forward primer	Reverse primer
CA9	human	NM_001216	5′- gggtgtcatctggactgtgtt -3′	5′- cttctgtgctgccttctcatc -3′
NDRG1	human	NM_006096	5′- atgtacccctccatggatca -3′	5′- tgtggaccacttccacgtta -3′
L28	human	NM_000991	5′- gcaattccttccgctacaac -3′	5′- tgttcttgcggatcatgtgt -3′
FIH	human	NM_017902	5′- cagttcgagtgcctctac -3′	5′ – atggccactttctgatgagc -3′
Hif1a	mouse	NM_010431	5′- ggttccagcagacccagtta -3′	5′- aggctccttggatgagcttt -3′
Hif2a	mouse	NM_010137	5′- taaagcggcagctggagtat -3′	5′- actgggaggcatagcactgt -3′
Bnip3	mouse	NM_009760	5′- gctcccagacaccacaagat -3′	5′- tgagagtagctgtgcgcttc -3′
Ca9	mouse	NM_139305	5′- gctgtcccatttggaagaaa -3′	5′- ggaaggaagcctcaatcgtt -3′
Glut1	mouse	NM_011400	5′- tctctgtcggcctctttgtt -3′	5′- gcagaagggcaacaggatac -3′
Pdk1	mouse	NM_172665	5′- ggcggctttgtgatttgtat -3′	5′- acctgaatcgggggataaac -3′
Phd1	mouse	NM_053208	5′- ttgcctgggtagaaggtcac -3′	5′- gctcgatgttggctaccact -3′
Phd2	mouse	NM_053207	5′- agccatggttgcttgttacc -3′	5′- ctcgctcatctgcatcaaaa -3′
Phd3	mouse	NM_028133	5′- caacttcctcctgtccctca -3′	5′- ggctggacttcatgtggatt -3′
Ndrg1	mouse	NM_010884	5′- tcaagatggcagactgtgga -3′	5′- gttgggggtgatgttgagac -3′
Sod1	mouse	NM_011434	5′- ccagtgcaggacctcatttt -3′	5′- cacctttgcccaagtcatct -3′
Sod2	mouse	NM_013671	5′- ggccaagggagatgttacaa -3′	5′- gaaccttggactcccaca -3′
Glrx	mouse	NM_053108	5′- aacaacaccagtgcgattca -3′	5′- atctgcttcagccgagtcat -3′
Ednrb	mouse	NM_007904	5′- cagtcttctgcctggtcctc -3′	5′- ggactgcttttcctcaaacg -3′
Mmp3	mouse	NM_010809	5′- ctatacgagggcacgaggag -3′	5′- ccacccttgagtcaacacct -3′
Svct1	mouse	NM_011397	5′- tctttggcctcacactaccc -3′	5′- tccttttttaccatgcccatc -3′
Svct2	mouse	NM_018824	5′- tgccaggaagggtgtacttc -3′	5′- ccggtaccaaatatgccatc -3′
Epo	mouse	NM_007942	5′- ggccatagaagtttggcaag -3′	5′- cctctcccgtgtacagcttc -3′
S12	mouse	NM_011295	5'- gaagctgccaaagccttaga -3'	5'- aactgcaaccaaccaccttc -3'

Author Manuscript II published in Blood, 2011 Feb 23. [Epub ahead of print].
PMID: 21346252

Table S2. Blood parameters of Gulo$^{-/-}$ mice. All mice received ascorbate free provender for 5 weeks. Control animals received vitamin C via their drinking water. Heparinized whole blood samples were collected from mice kept at ambient oxygen tension or in a hypoxic environment (8% oxygen for 24 hours) by cardiac puncture. The values represent mean values ± SD of at least three animals per group as indicated.

	20% oxygen [Fi O_2]		**8% oxygen [Fi O_2]**	
	+Asc (n=3)	**-Asc (n=4)**	**+Asc (n=4)**	**-Asc (n=5)**
Hemoglobin(g/dl)	12.57±0.6807	13.43±0.3304	12.33±2.022	12.20±1.030
Hematocrit (%)	40.23±1.528	42.88±0.3862	40.68±6.247	39.72±3.492
Erythrocytes (10^6/µl)	8.777±0.5724	9.250±0.09019	8.625±1.275	8.496±0.6687
Retikulocytes (10^3/µl)	260.3±42.34	227.0±21.46	295.3±106.4	273.2±53.18

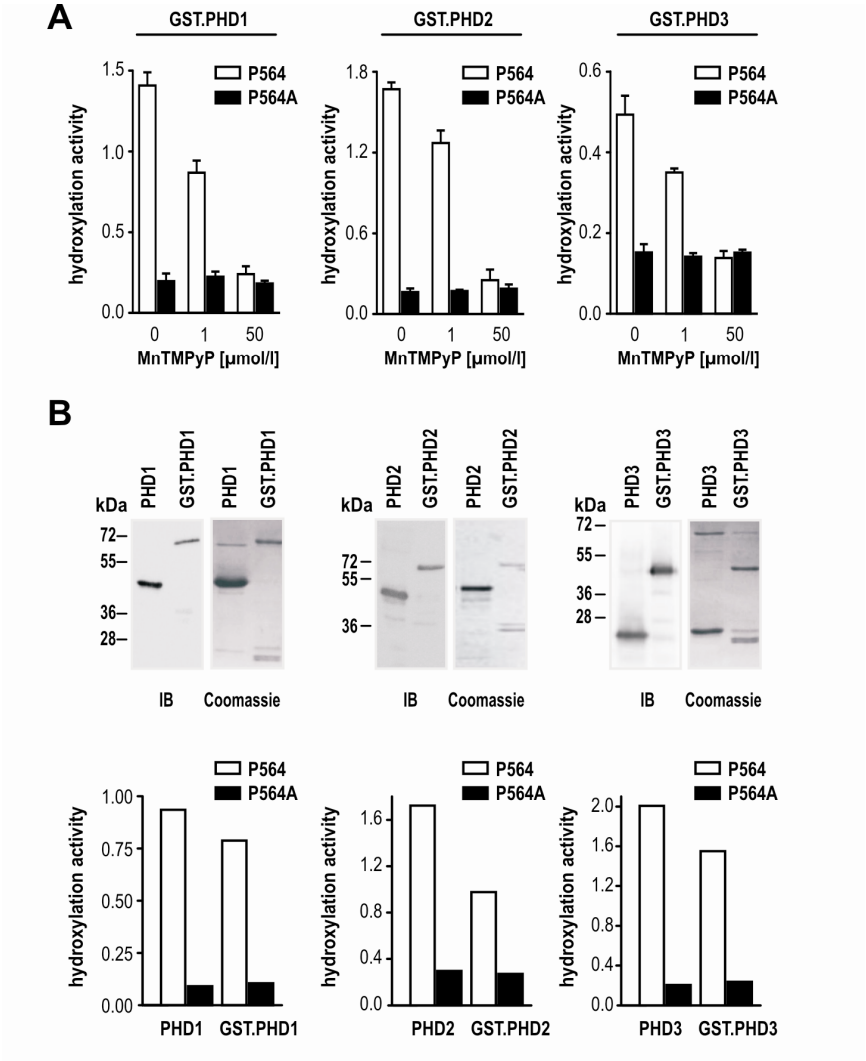

Fig. S1. Inhibition of PHDs by MnTMPyP and *in vitro* hydroxylation activity of untagged PHD1-3. (A) Inhibition of PHD1-3 hydroxylation activity by Mn(III) tetrakis (1-methyl-4-pyridyl) porphyrin pentachloride (MnTMPyP) measured by an *in vitro* hydroxylation assay. The presence of 50 µM MnTMPyP completely abolished hydroxylase activity of all three PHDs. Shown are mean values ± SEM of an experiment performed in triplicates. (B) Comparison of the purity (upper panels. IB, immunoblotting; Coomassie, protein staining by Coomassie blue) and hydroxylation activity (lower panels) of GST-tagged and untagged PHD1-3 enzyme preparations. Representative experiments performed in duplicates are shown. A Gateway-Technology compatible expression vector for GST fusion proteins bearing the PreScission protease cleavage site was generated by introducing a Leu-Glu-Val-Leu-Phe-Gln-Gly-Pro peptide into pDEST20 (Invitrogen) by site-directed mutagenesis. The C201S mutation was introduced into wild-type PHD2 by site-directed mutagenesis. PHD1, PHD2 and PHD3 expression vectors were generated by homologous recombination with respective Entry vectors (Invitrogen). For expression of recombinant proteins, Sf9 cells were infected with baculovirus stock and cultured in Grace's insect medium at 27ºC in a humidified incubator for 96-110 hours. Cells were collected by centrifugation and lysed in ice-cold 0.1% NP-40, 10 mM Tris-HCl pH 7.5, 100 mM NaCl, 100 mM glycine and 10 mM DTT. Cleared lysates were

incubated with PBS-equilibrated GSH-sepharose beads (GE Healthcare) for 2 hours at 4°C with gentle agitation. For cleavage, beads were washed twice with PBS and equilibrated twice with PreScission cleavage buffer (50 mM Tris-Cl pH 7.0, 150 mM NaCl). PreScission protease (GE Healthcare) was added to the protein-bound glutathione-sepharose beads (80 U in 960 ml cleavage buffer per 1 ml of bead volume) and incubated for 5 hours at 4ºC.

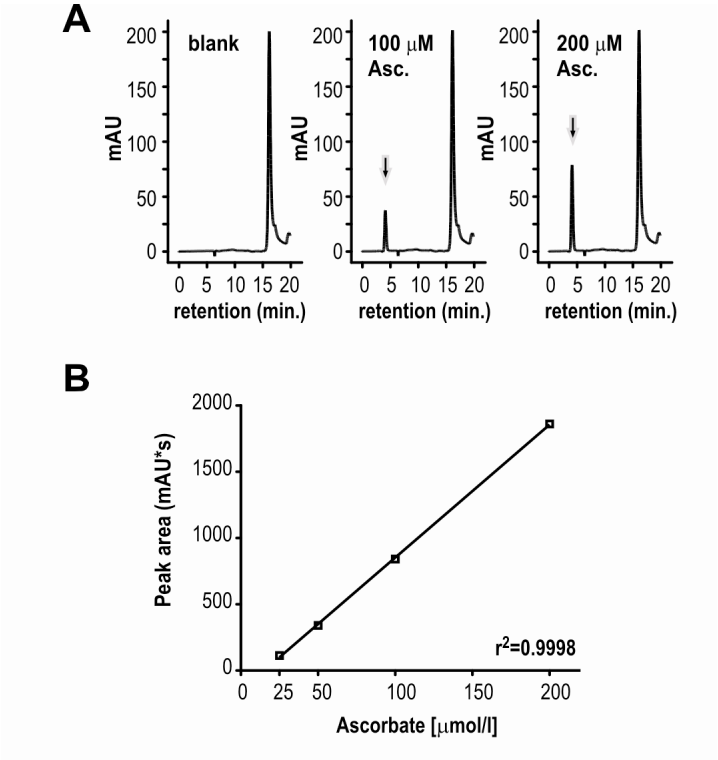

Fig. S2. HPLC determination of ascorbate concentrations. (A) Exemplary chromatograms derived from samples of blank (60 mM phosphoric acid, left), or spiked standard solutions containing 100 µM (middle) and 200 µM (right) ascorbate, respectively. Probes were loaded on a Nucleosil C18 column and eluted applying an acetonitrile gradient (0-60%). Arrows indicate the position of the peaks specific for ascorbate. (B) Standard curve as obtained after plotting the peak areas of ascorbate elutions (given in arbitrary units; AU) against known ascorbate concentrations in spiked samples. Regression analysis was performed using Prism 4.0 GraphPad software.

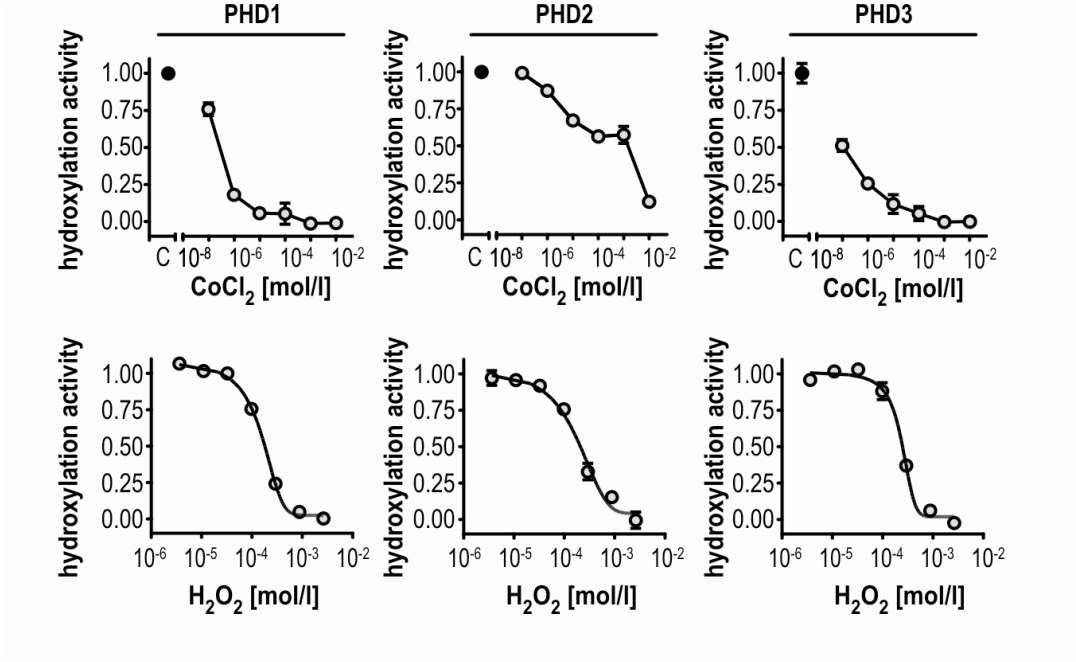

Fig. S3. $CoCl_2$ and H_2O_2 inhibit the *in vitro* hydroxylation activity of PHD1-3.
Dose-dependent inhibition of PHD activity by $CoCl_2$ (upper panel) and H_2O_2 (lower panel). Shown are mean values ± SEM of a representative experiment performed in triplicates.

8. Book chapter: Antioxidants and PHD oxygen sensing function

Katarzyna J. Nytko, Daniel P. Stiehl, Roland H. Wenger

Institute of Physiology and Zürich Center for Integrative Human Physiology ZIHP, University of Zürich, CH-8057 Zürich, Switzerland

Running title:

ANTIOXIDANTS AND PHD OXYGEN SENSOR FUNCTION

Significance:

- Like the collagen prolyl-4-hydroxylases, oxygen-sensing HIF prolyl-4-hydroxylases (PHDs) require vitamin C for their enzymatic activity
- Vitamin C deficiency leads to scurvy, raising the question whether it also affects oxygen sensing?
- There is evidence that antioxidants are essential to PHD function, but not necessarily vitamin C
- Consequently, antioxidants play important roles in HIF-dependent physiological and pathophysiological processes
- Detailed knowledge of antioxidant function will be necessary for the design of therapies of hypoxia-related diseases such as cancer

Correspondence:
Daniel P. Stiehl or Roland H. Wenger,
Institute of Physiology,
University of Zürich,
Winterthurerstrasse 190,
CH-8057
Zürich, Switzerland.
E-mail:roland.wenger@access.uzh.ch

Antioxidants and reactive oxygen species (ROS) are powerful modulators of many cellular and systemic pathways involved in physiological and pathophysiological processes of the human body. ROS are continuously generated in living cells and normally excessive production of ROS is antagonized by a number of antioxidative mechanisms. Through evolution cells acquired mechanisms to fight destructive effects of ROS on DNA, proteins and lipids by developing systems that either enzymatically remove ROS (superoxide dismutase, catalase, glutathione peroxidase etc.) or act as direct acceptors of unpaired electrons from ROS, thereby "scavenging" their harmful effects (carotenoids, vitamins C and E, glutathione etc.). Many diseases including cancer, atherosclerosis or inflammatory disorders are accompanied by an imbalanced antioxidative defense, resulting in severe damage to intra- and extracellular compartments ultimately promoting cell death or transformation. Besides their rather unspecific protective effects, many antioxidants serve as co-factors or reducing agents for numerous metal-containing cellular enzymes necessary for their designated function. As such, many enzymes require vitamin C for optimal activity, with collagen prolyl-4-hydroxylases (C-P4Hs) being the first described (Tuderman et al., 1977). Vitamin C further functions as a co-factor of other iron(II)-dependent dioxygenases as well as copper(I)-dependent monooxygenases involved in dopamine and tyrosine metabolism, carnitine synthesis, DNA repair, oxygen sensing and many others (Linster and Van Schaftingen, 2007; Mandl et al., 2009).

1. Ascorbate as a co-factor for prolyl-4-hydroxylases

Ascorbic acid is a water-soluble vitamin that can be found in cells either in the reduced form (ascorbate), as free radical (semidehydroascorbate) or fully oxidized as dehydroascorbate (DHA). At physiological pH, the most abundant form of ascorbic acid is ascorbate (Corti et al., 2010). Ascorbate and DHA are transported into cells by sodium-dependent transporters (SVCT) and glucose transporters (GLUT), respectively (Corti et al., 2010). Intracellular DHA can be reduced to ascorbate by enzymatic and non-enzymatic processes, mostly utilizing glutathione-dependent pathways. Interestingly, primates, guinea pigs and some species of bats and paseriform birds lost the ability to synthesize vitamin C due to a mutation of L-gulono-1,4-lactone oxidase (Gulo) which catalyses the final step of ascorbate synthesis (Linster and Van Schaftingen, 2007; Nishikimi et al., 1988). Other vertebrates *de novo* synthesize ascorbate from D-glucuronate in the liver (mammals) or in the kidney (fish, amphibians and reptiles) (Linster and Van Schaftingen, 2007). Since ascorbate is an essential co-factor of C-P4Hs which hydroxylate proline residue to stabilize the collagen triple helix structure, persistent ascorbate deficiency results in disassembly of connective tissue structures, a common symptom of the nowadays rare disease scurvy. Scurvy is further characterized by apathy, weakness, bleeding gums and external and internal hemorrhages, which in severe cases often led to death (Baron, 2009). The disease occurred endemically amongst sailors at sea having no access to fresh food for a long period of time, and was first described by the French explorer Jacques Cartier in the16th century (Martini, 2002). More than 200 years later the Scottish physician James Lind discovered that lemon juice can prevent the symptoms of the disease and seamen were subsequently provided with extracts of citrus fruits to treat scurvy (Lind, 1753). Until the discovery of vitamin C in 1932 by the later Nobel laureate Albert Szent-Györgyi, the reason for scurvy remained enigmatic and it took another 35 years until it finally could be established that vitamin C is actually required for full activity of C-P4Hs (Hutton et al., 1967; Li and Schellhorn, 2007). As ascorbate is present in almost all types of diets worldwide, the full picture of scurvy became very rare nowadays.

HIF prolyl-4-hydroxylases (PHDs) belong to the same class of enzymes as C-P4H, namely 2-oxoglutarate and non-heme iron(II)-dependent dioxygenases (EC 1.14.11.2). The prime substrate for C-P4H are prolyl residues within the collagen

molecule while two specific prolines of the oxygen susceptible α-subunits of hypoxia-inducible factors (HIFs) are targeted by PHDs (Epstein et al., 2001; Ivan et al., 2001; Jaakkola et al., 2001). So far three isoforms of oxygen sensing PHDs have been described in humans, namely PHD1, PHD2 and PHD3. These proteins are encoded by the human *EGLN2, EGLN1* and *EGLN3* genes, respectively. The proteins differ in size, intracellular localization and tissue distribution (Metzen et al., 2003; Stiehl et al., 2006; Wenger et al., 2009). Under experimental *in vitro* conditions, preferences of each PHD isoform for the two target proline substrates within the HIF-α molecule have been observed (Appelhoff et al., 2004; Chan et al., 2005). A new putative prolyl-4-hydroxylase was identified in the endoplasmic reticulum which, when overexpressed, can suppress HIF activity, too (Koivunen et al., 2007; Oehme et al., 2002). However, further studies must be performed to elucidate the physiological function of this isoform. Moreover, an asparaginyl hydroxylase termed factor inhibiting HIF (FIH) hydroxylates a C-terminal Asn-residue of HIF-α subunits in an oxygen-dependent manner, thereby regulation co-factor recruitment and HIF's transcriptional activity (Hewitson et al., 2002).

Structurally, C-P4Hs form α2β2 tetramers whereas soluble PHD2 was preferentially found as α-monomers (McDonough et al., 2006; Myllyharju, 2008). PHD3 can form homo and hetero complexes with other PHDs, and PHD1 and PHD2 can form homo-dimers (Nakayama et al., 2007). Interestingly, PHD1-3 share 42-59% sequence identity but only little similarity with the primary structure of C-P4H, though critical residues involved in binding of iron(II) and 2-oxoglutarate are conserved between the two subclasses (Myllyharju, 2008). Both, C-P4Hs and PHDs require oxygen, 2-oxoglutarate, iron (II) and/or ascorbate for *in vitro* activity, however they differ in *Km* values for these compounds (Hirsilä et al., 2003). Interestingly, the *Km* value of ascorbate for C-P4H is higher (300-370 μM) than for PHDs (140-180 μM), suggesting different dependency of these enzymes on ascorbate supplementation (Nagel et al., 2010). In contrast to 2-oxoglutarate and iron(II), which are tightly bound to PHDs, ascorbate did not co-purify with PHD2, indicating a rather labile and transient interaction between the enzyme and ascorbate (McNeill, Flashman et al. 2005). The actual role of ascorbate in the reaction catalyzed by PHDs is still a matter of debate: it has been postulated for C-P4Hs that ascorbate reduces ferryl iron(IV) intermediates to the active ferrous form. However, a similar function in the

reaction cycle catalyzed by PHDs still awaits formal proof (de Jong et al., 1982; Kaelin and Ratcliffe, 2008). It has been shown for C-P4H that ascorbate is not consumed stoichiometrically during proline hydroxylation, and the enzymes can perform a number of reaction cycles without ascorbate (Myllyharju, 2008). Yet, in the absence of a proline hydroxy-acceptor substrate, C-P4H can catalyze uncoupled reactions characterized by decarboxylation of 2-oxoglutarate to succinate where ascorbate is then consumed stoichiometrically and serves as an alternative oxygen acceptor (Myllylä, Majamaa et al. 1984; Myllyharju 2008).

Among the factors that stabilize HIF-1α protein levels via ROS generation are angiotensin II and thrombin. Angiotensin II–induced generation of ROS (hydrogen peroxide) decreases intracellular ascorbate levels and thus leads to HIF-1α stabilization. Interestingly, ascorbate prevented ROS-induced HIF-1α under normoxic but not hypoxic conditions (Page et al., 2008). The role of ascorbate in modulation of HIF-1α protein levels has also been investigated in primary umbilical vein endothelial cells and in skin fibroblasts (Vissers et al., 2007). Addition of ascorbate to normoxic, hypoxic and cobalt chloride treated primary umbilical vein endothelial cells and skin fibroblasts cells decreased HIF-1α protein levels. As concluded in this study, normoxic cells showed stabilization of HIF-1α protein explained by the lack of ascorbate in the culture medium. However, the medium was supplemented with 20% FCS which might also be a source of ascorbate.

2. Ascorbate–independent prolyl-4-hydroxylase function

Reducing equivalents for functional HIF-α hydroxylation do not seem to be limited to ascorbate as other antioxidants (e.g. reduced glutathione and DTT) independently support PHD activity *in vitro* and *in vivo* (Flashman et al., 2010, Nytko et al., unpublished data). Studies on the role of ascorbate and other reducing agents on PHD2 activity *in vitro* revealed that ascorbate can be substituted by its structural analogues if they contain the ene-diol portion conferring reducing properties (Flashman et al., 2010). Furthermore, reduced glutathione (GSH) and dithiothreitol (DTT) at high concentrations could partially replace ascorbate in *in vitro* hydroxylation reactions catalysed by PHDs (Flashman et al., 2010). GSH further enhanced hydroxylation of an artificial consensus ankyrin peptide by FIH, which

indicates its activating function also for other hydroxylases involved in oxygen sensing (Flashman et al., 2010).

To study the importance of vitamin C functions *in vivo*, a genetically modified mouse model incapable of synthesizing ascorbate has been generated. Animals with a bi-allelic disruption of the *Gul*o gene (Gulo$^{-/-}$) developed scurvy-like symptoms after 5 weeks of ascorbate withdrawal, evidenced by loss of body weight, internal hemorrhages, and disruption of elastic laminae building vessel walls (Maeda et al., 2000). When Gulo$^{-/-}$ mice were kept on an ascorbate-free diet for more than 6 weeks, the animals die from aortic rupture, pointing out the absolute requirement for vitamin C (Maeda et al., 2000). Partially contradictory to this finding, vitamin C-deficient Gulo$^{-/-}$ mice with a different genetic background still showed ascorbate-independent skin collagen and proline hydroxylation, indicating that *in vivo* other factors might at least partially compensate for the lack of ascorbate (Parsons et al., 2006). Notably, administration of glutathione ester to ascorbate-deficient guinea pigs could delay scurvy symptoms, further supporting the *in vivo* redundancy of intracellular antioxidants in the process of collagen hydroxylation (Martensson et al., 1993). Interestingly, neutrophils known to generate high levels of ROS to execute their pro-inflammatory function had defective apoptosis and increased HIF-1α levels when isolated from ascorbate depleted Gulo$^{-/-}$ mice (Vissers and Wilkie, 2007). Such observations may provide evidence for cell-type specific requirements of ascorbate in maintaining basal HIF activity, which is probably interlinked with the specific ROS burden in distinct cell types.

Whether ascorbate deficiency has an effect on other prolyl-4-hydroxylases *in vivo* at the systemic level is largely unknown. Recent work from our group showed that the physiologic response of ascorbate depleted Gulo$^{-/-}$ mice to oxygen deprivation was essentially normal when compared to vitamin C supplemented animals as evidenced by similar erythropoietin transcript and protein induction (Nytko et al., unpublished data). In line with this finding, ascorbate was also shown to be dispensable for carnitine synthesis *in vivo* (Furusawa et al., 2008). Taken together, these data strongly point towards a non-essential role of ascorbate for functional HIF-α prolyl-4-hydroxylation at cellular and systemic levels and the possibility of other antioxidants to compensate for its lack.

3. Regulation of PHD activity by redox factors

Interestingly, the β subunits of C-P4H have protein disulfide isomerase (PDI) activity which is required for the stable formation of the tetrameric enzyme (Kivirikko and Myllyharju, 1998). Since PHDs are active in the monomeric form (McDonough et al., 2006), a similar redox-sensitive mechanism is unlikey to exist for PHDs. We noted that recombinant PHD proteins purified from bacterial expression systems generally have low specific activity, with PHD3 being notoriously inactive, indicating the requirement of structural modulators absent in bacteria. In support of this notion, TriC and Morg1 chaperoins have been described to interact and stimulate the activity of PHD3 and overexpression of the bacterial GroEL/ES chaperoin enhanced the activity of the enzyme from bacterial preparations (Fedulova et al., 2007; Hopfer et al., 2006; Masson et al., 2004). However, it is unclear if these complex partners of PHD3 actively affect the protein structure by interference with intramolecular disulfide bonds.

For other antioxidants, e.g. the polyphenolic green tea ingredients gallic acid or its derivative n-propyl gallate, contradictory effects to ascorbate and glutathione on PHD acticity have been reported: Gallate, but not n-propyl gallate were claimed to inhibit PHDs *in vitro* while both compounds were stabilizing HIF-1α protein in rat heart muscle cells (Tsukiyama et al., 2006). Based on structural predictions of PHD2, inhibitory effects of gallate were attributed to the displacement of enzyme bound 2-oxoglutarate by gallate's carboxy moiety forming an ionic bond with Arg383, while its phenolate oxygen atoms chelate active centre bound iron(II) (Tsukiyama et al., 2006). However, data from our group using purified human PHD isoenzymes showed that either of these compounds was efficiently inhibiting all three PHDs in a dose-dependent manner *in vitro* (Nytko et al., 2007). The contradictory outcome of these experiments is likely explained by the use of crude cell extracts as enzyme source for *in vitro* HIF-α hydroxylation by Tsukiyama et al. We have shown previously that crude cell extracts catalyse non-PHD related turnover of 2-oxoglutarate to succinate and as such are inappropriate to determine specific PHD activity (Wirthner et al., 2007). Similarly, the plant-derived flavonoid quercetin has been shown to inhibit the activity of PHD2 *in vitro* probably involving by chelating active site bound iron(II) (Dao et al., 2009; Jeon et al., 2007). Other flavonoids, such as baicalein, luteolin and fisetin, increased HIF-1α protein levels in HeLa cells but only weakly induced HIF

transcriptional activity (Triantafyllou et al., 2008). Despite an assumed role in HIF-α protein stabilization, these compounds paradoxically blunted deferoxamine (DFX) induced HIF transcriptional activity, probably by interfering with MAPK-dependent activation of the HIF pathway (Triantafyllou et al., 2008). In a screen for small molecule inhibitors of PHD2, baicalein was independently identified as potent inhibitor of PHD2 *in vitro* but also reduced asparaginyl hydroxylation of HIF-1α by FIH (Cho et al., 2008). Inhibitory effects of bicalein on PHD activity could be abrogated by an excess of 2-oxoglutarate and iron(II), suggesting a competitive mechanism. Spectroscopic measurements provided indirect evidence for binding of bicalein to PHD2, which was specific for the iron(II) containing enzyme (Cho et al., 2008).

Clearly, the variety of opposite effects reported for antioxidants on the PHDs' hydroxylation activity is caused by the different modes of activatory or inhibitory action (Fig. 1). One common inhibitory mechanism involves chelation of metal co-factors required for PHD enzymatic activity, another mechanism might be the scavenging of some short-lived transition states of oxygen generated during the hydroxylation reaction cycle (Nytko et al., 2007). Moreover, phenolic antioxidants might contribute to the generation of reactive oxygen species such as hydrogen peroxide in cell culture models, subsequently inhibiting the cellular oxygen sensing pathway (Halliwell and Gutteridge, 2007).

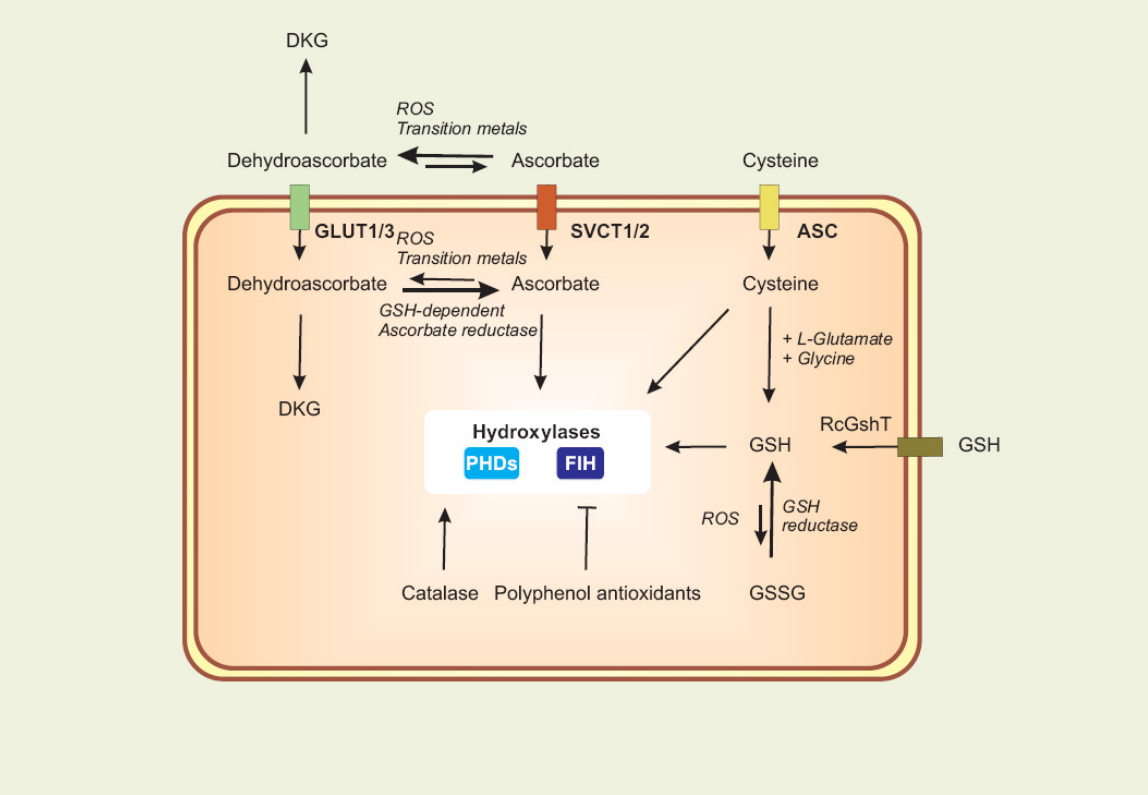

Fig. 1. Crosstalk between different antioxidants affecting the activity of HIF prolyl and asparaginyl hydroxylases. ROS, reactive oxygen species; PHDs, prolyl-4-hydroxylase domain proteins; FIH, factor inhibiting HIF; DKG, diketogulonate; GSH, glutathione; GSSG, oxidized glutathione dimer; GLUT, glucose transporter; SVCT,sodium-dependent vitamin C transporter; ASC, alanine, serine, cysteine preferring transport system; RcGshT, rat canalicular GSH transporter. Bold arrows indicate the preferred direction of the reaction.

3.1 Ascorbate and transition metals

Historically, cobalt(II) was described as a factor inducing erythropoiesis with medical applications in the treatment of anemia (Goldberg et al., 1988; Nagel et al., 2010; Wolf and Levy, 1954). However, mechanisms by which cobalt and other metals exert their inhibitory function on cellular oxygen sensing remained speculative. *In vitro* characterization of PHD isoenzymes confirmed that transition metals are potent inhibitors of PHD activity (Hirsilä et al., 2003). Given that PHD enzymes are iron(II)-dependent dioxygenases, it is tempting to speculate that transition metals act via replacement of ferrous iron in the catalytic center of the enzyme as proposed for soluble nickel compounds (Davidson et al., 2006). Moreover, chromium(VI),

nickel(II) and cobalt(II) have been proposed to regulate HIF-1α protein levels by depletion of intracellular ascorbate in lung cells while neither soluble nickel(II) nor cobalt(II) changed intracellular iron levels (Kaczmarek et al., 2007; Salnikow et al., 2004). Mechanistically, these metals are capable of catalyzing the oxidation of ascorbate to DHA, which can be enzymatically reverted to ascorbate within the cell or irreversibly oxidized to diketogulonate. Of note, *in vitro* oxidation of ascorbate by air is mainly catalyzed by copper(II) but oxidation is substantially slower by nickel(II) or cobalt(II) with a rate comparable to soluble iron(II), which does not inhibit PHD activity at all (Nytko et al., 2007). While this finding explains the PHD inhibiting effects observed for cupric copper (Martin et al., 2005; Nytko et al., 2007), the "ascorbate depletion" hypothesis by simple metal catalyzed oxidation should be marked by a touch of skepticism. Alternatively, nickel and cobalt could induce oxidative stress within the cell, as defined by the reduction of glutathione and malondialdehyde levels, thereby exhausting the intracellular ascorbate pool, instead of directly catalyzing ascorbate oxidation. Indeed, cobalt has been shown to induce oxidative stress in endothelial cells, which contributed to HIF-1α stabilization and was prevented by ascorbate treatment (Qiao et al., 2009). The increase in intracellular ROS and HIF-1α stabilization upon exposure to cobalt chloride in skeletal muscle cells was prevented by ascorbate treatment (Ciafre et al., 2007). Of note, cobalt chloride was found to robustly carbonylate recombinant PHD2 enzyme *in vitro* even in the complete absence of ascorbate, suggesting a direct redox interaction of the metal with iron-containing PHD enzymes (Nytko et al., unpublished data). Moreover, direct binding of cobalt to HIF-1α and subsequent inhibition of HIF-1α protein degradation by the pVHL ubiquitin ligase complex has been described, adding another layer of metal-dependent interference with the oxygen sensing pathway (Yuan et al., 2003). However, since 6xHis-HIF-2α proteins were used in above experiments, the binding of the cobalt to 6His-tagged HIF-2α could contribute to observed effect. Overall, in comparison to C-P4H and FIH, most transition metals were less effective inhibitors of PHDs, with PHD2 being particularly resistant to metal-induced regulation (Hirsilä et al., 2003).

3.2 Ascorbate and nitric oxide

It has been shown that nitric oxide can contribute to behavioural and developmental responses to hypoxia in *D. melanogaster* (Wingrove and O'Farrell, 1999). Moreover, as iNOS and eNOS (inducible and endothelial NO synthase, respectively) are direct HIF target genes (Coulet et al., 2003; Melillo et al., 1997; Palmer et al., 1998). NO signaling could provide an additional feedback loop to oxygen sensing via regulating PHD activity (Kaelin and Ratcliffe, 2008). However, a clear picture of how NO affects oxygen signaling has been blurred by numerous opposing reports showing either increase (Kimura et al., 2001) or decrease in HIF-1α protein levels and transcriptional activity upon NO treatment (Huang et al., 1999). A recent report offers an elegant explanation for this dilemma, providing evidence for bi-modal effects of NO on HIF-1α protein accumulation. First, NO administration inhibits PHD activity leading to a rapid induction of HIF-1α protein. With some delay, the cellular hydroxylation capacity increases by HIF-dependent *de novo* synthesis of PHD2 (and likely PHD3) protein, ultimately reducing HIF-1α levels and counteracting the initial NO response (Berchner-Pfannschmidt et al., 2007). A similar model of bi-phasic responses to the notoriously instable ROS intermediates might likewise explain the many controversial results reported for this molecule group with respect to oxygen signaling pathways. Adding more complexity, ascorbate has been shown to decrease the levels of HIF-1α protein in HUVECs cells induced by treatment with a NO donor (Muellner et al., 2010). Another study showed that NO-induced HIF-1α accumulates independently of PHD activity, proposing cystein nitrosylation of HIF-1α interfering with pVHL recruitment and asparagine hydroxylation to be responsible for its stabilization (Park et al., 2008). Adding even more complexity, ascorbate can stimulate NO release from intrinsic S-nitroso groups of the glypican-1 core protein (Mandl et al., 2009). Although this mechanism was primarily shown to play a role in NO-catalysed degradation of heparin sulphates, one could speculate about a similar role for ascorbate-induced NO generation in oxygen sensing (Mandl et al., 2009).

4. Expression of HIF-1α is affected by stress-activated pathways

Besides an obvious role of antioxidants in the reaction catalyzed by PHDs and thus stability of HIF-1α protein, antioxidants have also been associated with the regulation of HIF expression. Transcript levels of HIF-1α were shown to be increased by

reactive oxygen species involving activation of NFκB signaling (Bonello et al., 2007). Both hydrogen peroxide and thrombin (shown previously to induce ROS production) likewise induced HIF-1α mRNA levels, an effect that was blunted in presence of N-acetyl-cysteine (Belaiba et al., 2007; Bonello et al., 2007). Supporting these findings, the ubiquitous HIF-1α promoter contains a functional NFκB binding site, suggesting a role of this factor in the regulation of HIF-1α transcription (Bonello et al., 2007; Frede et al., 2006). Moreover, silencing of the antioxidant defense enzyme manganese-superoxide dismutase (Mn-SOD) increased both HIF-1α mRNA and protein levels following intracellular ROS levels under normoxic and hypoxic conditions (Sasabe et al., 2010). *In vivo* studies using mice lacking IκB kinase (IKK)-β confirmed that basal NFκB activity is linked with HIF-1α protein accumulation in the liver and brain of hypoxic animals (Rius et al., 2008).

Given the delicate balance of HIF-α production and proteasomal degradation, also regulated translation of HIF-α subunits has been suggested to affect the activity of the oxygen sensing pathway (Semenza, 2002; Yee Koh et al., 2008). Interestingly, the NO-donor NOC18 has been shown to increase the abundance of HIF-1α independent of its stability, which has been explained by concomitant activation of the protein translation regulatory mTOR/p70S6/eIF-4E pathway by NOC18 (Kasuno et al., 2004). Several other laboratories reported a role for PI3K and MAPK pathways in fine-tuning HIF-α accumulation and transactivation under normoxic and hypoxic conditions (Laughner et al., 2001; Richard et al., 1999; Sang et al., 2003; Stiehl et al., 2002; Zhong et al., 2000). Both pathways are tightly connected to external stimuli including oxidative stress, therefore a disbalance in redox homeostasis, particularly occuring during hypoxia followed by reoxygenation, might well affect HIF-1α translation. Among the small cellular redox proteins, thioredoxin (Trx) has also been implicated in HIF regulation, as reduced Trx could enhance HIF-1α protein levels and transcriptional activity (Huang et al., 1996; Welsh et al., 2002). It has later been resolved that Trx1 and Trx2 exert opposing regulatory functions on HIF-1α translation: while forced expression of cytosolic Trx1 enhanced HIF-1α protein levels in human embryonic kidney cells, the mitochondrial Trx2 attenuated hypoxic accumulation of HIF-1α. As an explanation, the authors referred to opposing effects of Trx1 and Trx2 on mitochondrial ROS production. Application of the mitochondria

targeting antioxidant MitoQ indeed abolished reduced HIF-1α accumulation in cells with exogenous Trx2 expression (Zhou et al., 2007).

Sirtuin 1 (Sirt1), a redox-sensitive deacetylase, physically interacts specifically with HIF-2α and increases transcription of HIF-2 dependent target genes such as *Epo* in cells and mice (Dioum et al., 2009). While the actual acetylating event remains enigmatic, the same study reported on hypoxically induced acetylation of endogenous HIF-2α which was detectable in the presence of deacetylase inhibitors only. Extending these observations, deacetylation of human HIF-1α at Lys674 by SIRT1 lead to impaired interaction of HIF-1α with p300 and consequently reduced HIF transcriptional activity (Lim et al., 2010). Interestingly, the plant derived polyphenolic antioxidant resveratrol has been described as potent regulator of sirtuin activity and hence could influence oxygen sensing *via* the HIF pathway (Howitz et al., 2003). Further strengthen the importance of acetylation in oxygen signaling, SIRT6 has been shown to function as a co-repressor of HIF-1α in the control of expression of several genes involved in glucose homeostasis (Zhong et al., 2010).

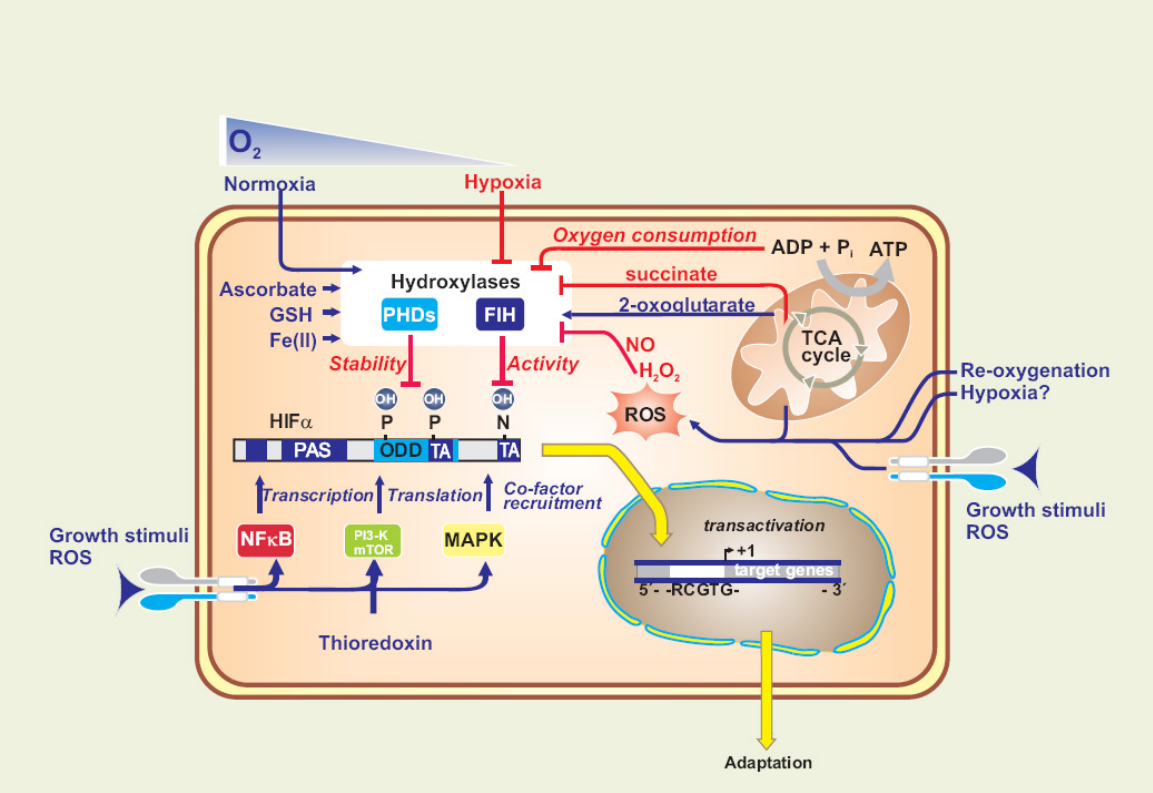

Fig. 2. Regulation of HIF transcription, translation, protein stability andtransactivationby reactive oxygen species and antioxidants. PHDs, prolyl-4-hydroxylase domain proteins; FIH, factor inhibiting HIF; HIF, hypoxia inducible factor; ROS, reactive oxygen species; NO, nitric oxide; GSH, glutathione; MAPK, mitogen activated protein kinase; NFkB, nuclear factor kappa light enhancer of activated B cells; mTOR, mammalian target of rapamycin; PI3-K, phosphatidylinositol 3-kinase; PAS, Per/Arnt/Sim; ODD, oxygen dependent degradation; TA, transactivation domain; TCA, tricarboxylic acid.

5. Oxidative stress and antioxidants in systemic oxygen sensing and disease

Following the instant response to cellular hypoxia and acute reprogramming of cell metabolism, HIFs also mediate middle- and long-term adaptational responses at the systemic level. HIF-1α protein stabilization by ROS has been discussed in the process of hypoxia-induced pulmonary vascular leakage, which causes pulmonary edema as described in mountain sickness and acute respiratory distress syndrome (ARDS) (Irwin et al., 2009). Cultured bovine pulmonary artery endothelial cell monolayers exposed to 3% O_2 for 24 hours showed increased permeability as measured by FITC-labeled albumin diffusion (Irwin et al., 2009). An antioxidative cocktail containing ascorbic acid, glutathione and α-tocopherol reduced the permeability when added to

the cell monolayer, along with decreased hydrogen peroxide production and HIF-1α protein levels in these cells. Similar results were obtained in mice exposed to high altitude (18000 ft ≈ 5500 m above sea level) for 24 hours. The animals showed hypoxia-induced pulmonary vascular leakage and responded with increased HIF-1α and VEGF protein levels in pulmonary vessels, which could be reverted upon administration of antioxidants (Irwin et al., 2009). In human volunteers exposed to an altitude of 4300 m above sea level, corresponding to roughly 12% inspiratory oxygen concentration, HIF-1α protein levels and HIF-1 DNA binding were enhanced in circulating leukocytes and total glutathione levels in the plasma were strikingly decreased by 35% (Tissot van Patot et al., 2009). Such data might suggests that under hypoxic conditions the glutathione pool, serving as the major physiological antioxidant in humans, gets exhausted, which in turn could potentiate hypoxic HIF-1α activation.

The role of antioxidants in erythropoiesis has been studied in cell culture models *in vitro* as well as *in vivo*. EPO secretion from isolated rat kidneys perfused with an arterial pO_2 of 35 mm Hg was increased upon treatment with vitamins A, E and C (Jelkmann et al., 1997). Yet, vitamins E and C failed to increased Epo secretion from the human hepatoma cell lines Hep3B and HepG2 (Jelkmann et al., 1997). Oxidative stress has also been observed in haematopoietic disorders. Patients suffering for inherited forms of anemia such as sickle cell anemia, thalassemia and glucose-6-phosphate-dehydrogenase deficiency, levels of certain antioxidants (predominantly vitamins E and C) are often decreased (Chan et al., 1999).

Physiological wound healing requires oxygen for an optimal healing process (Schreml et al., 2010). ROS produced during wound healing have been discussed to serve as signaling molecules in regulating cytokine release and cell proliferation, while they also contribute to killing harmful bacteria in the wound environment (Schreml et al., 2010). On the other hand, oxygen deprivation is supportive in wound healing as it stimulates VEGF expression and neovascularization (Hopf and Rollins, 2007; Scheid et al., 2000). Importantly, levels of ascorbate, vitamin E and glutathione are decreased in healing skin wounds and reduced level of these antioxidants correlate with a delay in the healing process (Schafer and Werner, 2008). Consequently, detoxifying enzymes, such SODs, catalase and glutathione peroxidase and others, are involved in wound healing and responsible for maintaining a cellular redox balance

(Schafer and Werner, 2008). Very likely, hypoxia, ROS and antioxidative pathways interact with each other to contribute to successive wound healing, though experimental proof of such a hypothesis is required.

Antioxidants play also important role in aging and aging is associated with increased oxidative stress (Rockenfeller and Madeo, 2010). Mice lacking senescence marker protein 30, which functions as gluconolactonase and participates in ascorbate synthesis, displayed symptoms of scurvy and shortage in lifespan (Kondo et al., 2006). Moreover, ascorbate was shown to prevent premature aging in mice displaying the phenotype of Werner syndrome due to lack of helicase of WRN homolog involved in DNA repair (Massip et al., 2009). Whether there is a direct link between HIF and antioxidants in context of aging remains to be investigated.

6. Antioxidants in HIF-mediated tumorigenesis

In the 1970s, Linus Pauling reported that ascorbate can prolong the survival time of terminally ill cancer patients (Cameron and Pauling, 1976; Cameron and Pauling, 1978). Moreover, he claimed that ascorbate is applicable in the prevention of common cold and schizophrenia (Pauling, 1971; Pauling, 1977). However, his hypotheses did not find general approval by the scientific community and other studies failed to confirm these results (Creagan et al., 1979; Moertel et al., 1985). An impressive number of studies on ascorbate and cancer has been performed since that time and the general idea of using ascorbate in cancer treatment has experienced a renaissace in recent years: growing a collection of tumor cell lines as subcutaneous tumors in mice revealed that ascorbate administed at “pharamcological doses” could decrease the growth of aggressive tumor xenografts (Chen et al., 2008). In these studies, and somehow paradoxically, ascorbate has been proposed to function as a pro-drug which facilitates the generation of hydrogen peroxide in a Fenton-like reaction and selectively kills cancer cells (Chen et al., 2005; Chen et al., 2007). Of note, millimolar doses of ascorbate can be achieved only by intravenous application of ascorbate and the findings have been perceived not without controversy (Borst, 2008; Frei and Lawson, 2008). Moreover, such studies do not consider the role of HIF-1/2α in cancer progression. It is known that high expression of HIF-1/2α correlates with high mortality of cancer patients, especially in the case of solid tumors (Semenza, 2010). Little is known, however, about the correlation between ascorbate levels in patients

and cancer progression in the context of HIF-1/2α expression and stability. It has been shown for endometrial cancers only that low ascorbate levels in cancer tissue is associated with increased HIF-1α activity and elevated expression of HIF-target genes (VEGF, GLUT-1 and BNIP3) (Kuiper et al., 2010). Endometrial cancers are associated with high expression of HIF-1/2α and increased angiogenesis, which is linked to poor prognosis of the patients (Sivridis et al., 2002). Therefore, finding a way to overcome HIF-driven effects in these tumors by antioxidants could be a potential therapy target. Ascorbate can suppress HIF-1α protein levels and secretion of VEGF in pancreatic tumor in athymic mice, but ascorbate could reduce tumor size only at early and middle stage of cancer progression (Chen et al., 2009). In cancer cells *in vitro*, ascorbate supplementation could reduce HIF-1α protein levels in normoxic prostate cancer cells, which have high basal levels of this protein (Knowles et al., 2003). Moreover, ascorbate decreased HIF-1α protein levels stimulated by insulin and insulin growth factor I (IGF-1) in human breast cancer cells (Knowles et al., 2003). Interestingly, ascorbate had no effect on hypoxia-induced HIF-1α protein levels. Since iron(II) is a crucial co-factor for the activity of PHDs, its depletion from cells results in the stabilization of HIF-1α and activation of target genes. It has been shown that inhibition of transferrin receptor by monoclonal antibodies and thus decrease in iron uptake leads to the stabilization of HIF-1α and upregulation of its targets in cancer cells (Jones et al., 2006). These effects were suppressed by physiological concentration of ascorbate (25 μM). At this point, it is important to mention that DHA, the oxidized form of ascorbate, can be transported to cells by glucose transporters (GLUT 1, 3 and 4) (Corti et al., 2010). Interestingly, GLUT1 and GLUT3 are also HIF-target genes, thereofore DHA uptake could be increased in hypoxia providing an additional feedback loop mechanism to balance HIF homeostasis, especially in cancer cells.

HIF-1α can mediate the inhibitory effects of antioxidants on tumor growth also *in vivo*. Benign prostatic hyperplasia (BPH) as well as prostate cancer are associated with hypoxia and angiogenesis. Moreover, testosterone induces HIF-1α levels in prostate cells (Mabjeesh et al., 2003). Ascorbate could reduce HIF-1α protein levels induced by testosterone treatment and decreased VEGF expression in prostate cells (Li et al., 2009). Moroever, ascorbate delayed prostate growth induced by testosterone in rats (Li et al., 2009).

Ascorbate deficiency had no effect on proline hydroxylation and collagen production in the mammary tumor cells (4TI) grown in Gulo$^{-/-}$ mice (Parsons et al., 2006). In contrast, ascorbic acid deficiency delayed growth of Lewis lung carcinoma in Gulo$^{-/-}$ (Telang et al., 2007). However, HIF-1α protein levels were not altered between ascorbate-free and ascorbate-supplemented tumors (Telang et al., 2007). These opposing effects could be explained by the different genetic background of Gulo$^{-/-}$ mice used in these experiments (Parsons et al., 2006; Telang et al., 2007). P493 human B cell xenografts grown in mice treated with N-acetyl cysteine (NAC, 40 mM) were significantly smaller than those in non-treated animals (Gao et al., 2007). Since P493 cells ectopically expressing non-degradable HIF-1α protein were more resistant to NAC treatment, HIF-1α might be involved in these effects (Gao et al., 2007). Moreover, NAC did not affect chromosomal stability, therefore excluding ROS-induced genomic instability as the reason for tumor growth. Similar effects were observed in P493 tumor xenografts when mice obtained a high concentration of ascorbate with the drinking water (5 g/l) (Gao et al., 2007). NAC is an antioxidant itself but can also increase the intracellular pool of glutathione, since it is a precursor in the synthesis of this compound. Until recently, antioxidant effects on tumor growth were attributed to its ability to reduce antioxidative stress and therefore genomic instability (Sablina et al., 2005). For the first time these data suggested that antioxidants could also prevent or delay tumor growth via mechanism mediated by PHDs/HIF. Another protein linking HIF- and ROS-dependent pathways in cancer cells is REDD1, a transcriptional target of HIF. REDD1 is also known as RT801 and is involved in the regulation of cell survival as well as ROS production (Ellisen et al., 2002; Shoshani et al., 2002). Increased ROS production and tumorigenesis has been shown for REDD1$^{-/-}$ cells, both mediated by HIF-1α, which can be blocked by treating the cells/animals with ascorbate and N-acetyl cysteine (Horak et al., 2010). JunD is a member of the AP-1 family of transcription factors and its lack in cells causes increased hydrogen peroxide generation and reduced antioxidative defense. As a result, these cells have increased HIF-1α levels which promotes angiogenesis and survival of tumor cells (Gerald et al., 2004). These effects have been shown to be dependent on PHD2 activity, but reduced in JunD$^{-/-}$ cells by oxidation of iron (II) in the catalytic center of the enzyme. Interestingly, hydrogen peroxide concentrations and levels of HIF-1α proteins in JunD$^{-/-}$ cells were reduced by treatment with

ascorbate, cysteine and reduced glutathione. These observations once again emphasize the role of these antioxidants in maintaing PHD activity and HIF-1α protein levels, especially under oxidative stress conditions.

Besides ascorbate and glutathione (or N-acetyl-cysteine) also vitamin E has been shown to exert antitumorigenic effects. Tocotrienol (unsaturated vitamin E) reduced hypoxia induced VEGF secretion from human colorectal adenocarcinoma cells (DLD-1) (Shibata et al., 2008). Considering that HIF-1α mediated angiogenesis is one of the major steps in cancer progression, these observation suggest vitamin E as a putative antitumorigenic agent.

As mentioned above, copper, nickel and other transition metals can deplete ascorbate (and other co-factors) required for PHD activity, stabilize HIF-1α and hence act as carcinogenic factors. These effects play an important role especially in cancers of the respiratory tract, since exposure to nickel(II), chromium(VI) and cobalt(II) via inhalation or ingestion causes primarily lung injury (Salnikow and Kasprzak, 2005). Moreover, advanced stages of lung cancer display decreased levels of ascorbate, reduced glutathione and other antioxidants (Esme et al., 2008).

7. Future perspectives

Whether antioxidants will serve as anti-cancer drugs in the context of the PHD/HIF oxygen sensing pathway remains speculative. The complexity of HIF responses together with the fact that in some tumors HIF down- and up-regulation exerts differential effects must be considered in such a therapies. There is no doubt that HIF prolyl-4-hydroxylases require antioxidants for full activity. Whether this antioxidant is exclusively ascorbate, or if it can be replaced by other reducing compounds, needs to be elucidated. One has also to consider that there are three different PHD isoforms with partial redundancy but also distinct cellular and organ expression levels, inducibility and probably also function. Furthermore, the specificity of antioxidant action on cells is very low with many enzymes/pathways being regulated and therefore difficult to control. Another point to be considered is a concentration-dependent dual function of ascorbate, either serving as ROS scavenger or as pro-oxidant (Levine et al., 2009). The border between these two states is very tightly regulated and depends on many factors that are difficult to control *in vivo*. Moreover, the half-life of most antioxidants is very short with intermediate states which exert

differential effects. Thus, antioxidants can undergo rapid modifications/degradation while administered to organism. The route of administration of antioxidants must be also considered as well. In Linus Pauling's studies, patients received ascorbate both orally and intravenously and in the follow-up studies, which failed to confirm Pauling's hypotheses, ascorbate was only orally applied to cancer patients, potentially explaining the opposing effects. Intravenous administration of ascorbate can result in 70 to 100-fold higher plasma concentrations of this compound (Levine et al., 2009). Overall, antioxidants have been proven to play an important role in the oxygen sensing pathway on almost all levels of HIF regulation: expression, stability and activity. However, further studies are required to elucidate their application in diseases mediated by PHDs/HIF oxygen sensing pathway.

References:

Appelhoff RJ, Tian YM, Raval RR, Turley H, Harris AL, Pugh CW, Ratcliffe PJ, Gleadle JM (2004). Differential function of the prolyl hydroxylases PHD1, PHD2, and PHD3 in the regulation of hypoxia-inducible factor. J Biol Chem. 279(37): 38458-65.

Baron JH (2009). Sailors' scurvy before and after James Lind--a reassessment. Nutr Rev. 67(6): 315-32.

Belaiba RS, Bonello S, Zahringer C, Schmidt S, Hess J, Kietzmann T, Gorlach A (2007). Hypoxia up-regulates hypoxia-inducible factor-1alpha transcription by involving phosphatidylinositol 3-kinase and nuclear factor kappaB in pulmonary artery smooth muscle cells. Mol Biol Cell. 18(12): 4691-7.

Berchner-Pfannschmidt U, Yamac H, Trinidad B, Fandrey J (2007). Nitric oxide modulates oxygen sensing by hypoxia-inducible factor 1-dependent induction of prolyl hydroxylase 2. J Biol Chem. 282(3): 1788-96.

Bonello S, Zahringer C, BelAiba RS, Djordjevic T, Hess J, Michiels C, Kietzmann T, Gorlach A (2007). Reactive oxygen species activate the HIF-1alpha promoter via a functional NFkappaB site. Arterioscler Thromb Vasc Biol. 27(4): 755-61.

Borst P (2008). Mega-dose vitamin C as therapy for human cancer? Proc Natl Acad Sci U S A. 105(48): E95; author reply E96.

Cameron E, Pauling L (1976). Supplemental ascorbate in the supportive treatment of cancer: Prolongation of survival times in terminal human cancer. Proc Natl Acad Sci U S A. 73(10): 3685-9.

Cameron E, Pauling L (1978). Supplemental ascorbate in the supportive treatment of cancer: reevaluation of prolongation of survival times in terminal human cancer. Proc Natl Acad Sci U S A. 75(9): 4538-42.

Chan AC, Chow CK, Chiu D (1999). Interaction of antioxidants and their implication in genetic anemia. Proc Soc Exp Biol Med. 222(3): 274-82.

Chan DA, Sutphin PD, Yen SE, Giaccia AJ (2005). Coordinate regulation of the oxygen-dependent degradation domains of hypoxia-inducible factor 1 alpha. Mol Cell Biol. 25(15): 6415-26.

Chen C, Sun J, Liu G, Chen J (2009). Effect of small interference RNA targeting HIF-1alpha mediated by rAAV combined L: -ascorbate on pancreatic tumors in athymic mice. Pathol Oncol Res. 15(1): 109-14.

Chen Q, Espey MG, Krishna MC, Mitchell JB, Corpe CP, Buettner GR, Shacter E, Levine M (2005). Pharmacologic ascorbic acid concentrations selectively kill cancer cells: action as a pro-drug to deliver hydrogen peroxide to tissues. Proc Natl Acad Sci USA 102(38): 13604-9.

Chen Q, Espey MG, Sun AY, Lee JH, Krishna MC, Shacter E, Choyke PL, Pooput C, Kirk KL, Buettner GR, Levine M (2007). Ascorbate in pharmacologic concentrations selectively generates ascorbate radical and hydrogen peroxide in extracellular fluid in vivo. Proc Natl Acad Sci U S A. 104(21): 8749-54.

Chen Q, Espey MG, Sun AY, Pooput C, Kirk KL, Krishna MC, Khosh DB, Drisko J, Levine M (2008). Pharmacologic doses of ascorbate act as a prooxidant and decrease growth of aggressive tumor xenografts in mice. Proc Natl Acad Sci USA 105(32): 11105-9.

Cho H, Lee HY, Ahn DR, Kim SY, Kim S, Lee KB, Lee YM, Park H, Yang EG (2008). Baicalein induces functional hypoxia-inducible factor-1alpha and angiogenesis. Mol Pharmacol. 74(1): 70-81.

Ciafre SA, Niola F, Giorda E, Farace MG, Caporossi D (2007). CoCl(2)-simulated hypoxia in skeletal muscle cell lines: Role of free radicals in gene up-regulation and induction of apoptosis. Free Radic Res. 41(4): 391-401.

Corti A, Casini AF, Pompella A (2010). Cellular pathways for transport and efflux of ascorbate and dehydroascorbate. Arch 500(2): 107-15.

Coulet F, Nadaud S, Agrapart M, Soubrier F (2003). Identification of hypoxia-response element in the human endothelial nitric-oxide synthase gene promoter. J Biol Chem. 278(47): 46230-40.

Creagan ET, Moertel CG, O'Fallon JR, Schutt AJ, O'Connell MJ, Rubin J, Frytak S (1979). Failure of high-dose vitamin C (ascorbic acid) therapy to benefit patients with advanced cancer. A controlled trial. N Engl J Med. 301(13): 687-90.

Dao JH, Kurzeja RJ, Morachis JM, Veith H, Lewis J, Yu V, Tegley CM, Tagari P (2009). Kinetic characterization and identification of a novel inhibitor of hypoxia-inducible factor prolyl hydroxylase 2 using a time-resolved fluorescence resonance energy transfer-based assay technology. Anal Biochem. 384(2): 213-23.

Davidson TL, Chen H, Di Toro DM, D'Angelo G, Costa M (2006). Soluble nickel inhibits HIF-prolyl-hydroxylases creating persistent hypoxic signaling in A549 cells. Mol Carcinog. 45(7): 479-89.

de Jong L, Albracht SP, Kemp A (1982). Prolyl 4-hydroxylase activity in relation to the oxidation state of enzyme-bound iron. The role of ascorbate in peptidyl proline hydroxylation. Biochim Biophys Acta. 704(2): 326-32.

Dioum EM, Chen R, Alexander MS, Zhang Q, Hogg RT, Gerard RD, Garcia JA (2009). Regulation of hypoxia-inducible factor 2alpha signaling by the stress-responsive deacetylase sirtuin 1. Science. 324(5932): 1289-93.

Ellisen LW, Ramsayer KD, Johannessen CM, Yang A, Beppu H, Minda K, Oliner JD, McKeon F, Haber DA (2002). REDD1, a developmentally regulated transcriptional target of p63 and p53, links p63 to regulation of reactive oxygen species. Mol Cell. 10(5): 995-1005.

Epstein AC, Gleadle JM, McNeill LA, Hewitson KS, O'Rourke J, Mole DR, Mukherji M, Metzen E, Wilson MI, Dhanda A, Tian YM, Masson N, Hamilton DL, Jaakkola P, Barstead R, Hodgkin J, Maxwell PH, Pugh CW, Schofield CJ, Ratcliffe PJ (2001). C. elegans EGL-9 and mammalian homologs define a family of dioxygenases that regulate HIF by prolyl hydroxylation. Cell. 107(1): 43-54.

Esme H, Cemek M, Sezer M, Saglam H, Demir A, Melek H, Unlu M (2008). High levels of oxidative stress in patients with advanced lung cancer. Respirology. 13(1): 112-6.

Fedulova N, Hanrieder J, Bergquist J, Emren LO (2007). Expression and purification of catalytically active human PHD3 in Escherichia coli. Protein Expr Purif. 54(1): 1-10.

Flashman E, Davies SL, Yeoh KK, Schofield CJ (2010). Investigating the dependence of the hypoxia-inducible factor hydroxylases (factor inhibiting HIF and prolyl hydroxylase domain 2) on ascorbate and other reducing agents. Biochem 427(1): 135-42.

Frede S, Stockmann C, Freitag P, Fandrey J (2006). Bacterial lipopolysaccharide induces HIF-1 activation in human monocytes via p44/42 MAPK and NF-kappaB. Biochem J. 396(3): 517-27.

Frei B, Lawson S (2008). Vitamin C and cancer revisited. Proc Natl Acad Sci U S A. 105(32): 11037-8.

Furusawa H, Sato Y, Tanaka Y, Inai Y, Amano A, Iwama M, Kondo Y, Handa S, Murata A, Nishikimi M, Goto S, Maruyama N, Takahashi R, Ishigami A (2008). Vitamin C is not essential for carnitine biosynthesis in vivo: verification in vitamin C-depleted senescence marker protein-30/gluconolactonase knockout mice. Biol Pharm Bull. 31(9): 1673-9.

Gao P, Zhang H, Dinavahi R, Li F, Xiang Y, Raman V, Bhujwalla ZM, Felsher DW, Cheng L, Pevsner J, Lee LA, Semenza GL, Dang CV (2007). HIF-dependent antitumorigenic effect of antioxidants in vivo. Cancer Cell 12(3): 230-8.

Gerald D, Berra E, Frapart YM, Chan DA, Giaccia AJ, Mansuy D, Pouyssegur J, Yaniv M, Mechta-Grigoriou F (2004). JunD reduces tumor angiogenesis by protecting cells from oxidative stress. Cell. 118(6): 781-94.

Goldberg MA, Dunning SP, Bunn HF (1988). Regulation of the erythropoietin gene: evidence that the oxygen sensor is a heme protein. Science. 242(4884): 1412-5.

Halliwell B, Gutteridge JM (2007). Free radicals in biology and medicine. Oxford University Press, Oxford, p.184.

Hewitson KS, McNeill LA, Riordan MV, Tian YM, Bullock AN, Welford RW, Elkins JM, Oldham NJ, Bhattacharya S, Gleadle JM, Ratcliffe PJ, Pugh CW, Schofield CJ (2002). Hypoxia-inducible factor (HIF) asparagine hydroxylase is identical to factor inhibiting HIF (FIH) and is related to the cupin structural family. J Biol Chem. 277(29): 26351-5.

Hirsilä M, Koivunen P, Günzler V, Kivirikko KI, Myllyharju J (2003). Characterization of the human prolyl 4-hydroxylases that modify the hypoxia-inducible factor. J Biol Chem 278(33): 30772-80.

Hopf HW, Rollins MD (2007). Wounds: an overview of the role of oxygen. Antioxid Redox Signal. 9(8): 1183-92.

Hopfer U, Hopfer H, Jablonski K, Stahl RA, Wolf G (2006). The novel WD-repeat protein Morg1 acts as a molecular scaffold for hypoxia-inducible factor prolyl hydroxylase 3 (PHD3). J Biol Chem. 281(13): 8645-55.

Horak P, Crawford AR, Vadysirisack DD, Nash ZM, DeYoung MP, Sgroi D, Ellisen LW (2010). Negative feedback control of HIF-1 through REDD1-regulated ROS suppresses tumorigenesis. Proc 107(10): 4675-80.

Howitz KT, Bitterman KJ, Cohen HY, Lamming DW, Lavu S, Wood JG, Zipkin RE, Chung P, Kisielewski A, Zhang LL, Scherer B, Sinclair DA (2003). Small molecule activators of sirtuins extend Saccharomyces cerevisiae lifespan. Nature. 425(6954): 191-6.

Huang LE, Arany Z, Livingston DM, Bunn HF (1996). Activation of hypoxia-inducible transcription factor depends primarily upon redox-sensitive stabilization of its alpha subunit. J Biol Chem. 271(50): 32253-9.

Huang LE, Willmore WG, Gu J, Goldberg MA, Bunn HF (1999). Inhibition of hypoxia-inducible factor 1 activation by carbon monoxide and nitric oxide. Implications for oxygen sensing and signaling. J Biol Chem. 274(13): 9038-44.

Hutton JJ, Jr., Kaplan A, Udenfriend S (1967). Conversion of the amino acid sequence gly-pro-pro in protein to gly-pro-hyp by collagen proline hydroxylase. Arch Biochem Biophys. 121(2): 384-91.

Irwin DC, McCord JM, Nozik-Grayck E, Beckly G, Foreman B, Sullivan T, White M, T. Crossno J J, Bailey D, Flores SC, Majka S, Klemm D, van Patot MC (2009). A potential role for reactive oxygen species and the HIF-1alpha-VEGF pathway in hypoxia-induced pulmonary vascular leak. Free Radic Biol Med. 47(1): 55-61.

Ivan M, Kondo K, Yang H, Kim W, Valiando J, Ohh M, Salic A, Asara JM, Lane WS, Kaelin WG, Jr. (2001). HIFα targeted for VHL-mediated destruction by proline hydroxylation: implications for O2 sensing. Science 292(5516): 464-8.

Jaakkola P, Mole DR, Tian YM, Wilson MI, Gielbert J, Gaskell SJ, Kriegsheim A, Hebestreit HF, Mukherji M, Schofield CJ, Maxwell PH, Pugh CW, Ratcliffe PJ (2001). Targeting of HIF-α to the von Hippel-Lindau ubiquitylation complex by O2-regulated prolyl hydroxylation. Science 292(5516): 468-72.

Jelkmann W, Pagel H, Hellwig T, Fandrey J (1997). Effects of antioxidant vitamins on renal and hepatic erythropoietin production. Kidney Int. 51(2): 497-501.

Jeon H, Kim H, Choi D, Kim D, Park SY, Kim YJ, Kim YM, Jung Y (2007). Quercetin activates an angiogenic pathway, hypoxia inducible factor (HIF)-1-vascular endothelial growth factor, by inhibiting HIF-prolyl hydroxylase: a structural analysis of quercetin for inhibiting HIF-prolyl hydroxylase. Mol Pharmacol. 71(6): 1676-84.

Jones DT, Trowbridge IS, Harris AL (2006). Effects of transferrin receptor blockade on cancer cell proliferation and hypoxia-inducible factor function and their differential regulation by ascorbate. Cancer Res. 66(5): 2749-56.

Kaczmarek M, Timofeeva OA, Karaczyn A, Malyguine A, Kasprzak KS, Salnikow K (2007). The role of ascorbate in the modulation of HIF-1alpha protein and HIF-dependent transcription by chromium(VI) and nickel(II). Free Radic Biol Med. 42(8): 1246-57.

Kaelin WG, Jr., Ratcliffe PJ (2008). Oxygen sensing by metazoans: the central role of the HIF hydroxylase pathway. Mol Cell. 30(4): 393-402.

Kasuno K, Takabuchi S, Fukuda K, Kizaka-Kondoh S, Yodoi J, Adachi T, Semenza GL, Hirota K (2004). Nitric oxide induces hypoxia-inducible factor 1 activation that is dependent on MAPK and phosphatidylinositol 3-kinase signaling. J Biol Chem. 279(4): 2550-8.

Kimura H, Weisz A, Ogura T, Hitomi Y, Kurashima Y, Hashimoto K, D'Acquisto F, Makuuchi M, Esumi H (2001). Identification of hypoxia-inducible factor 1 ancillary sequence and its function in vascular endothelial growth factor gene induction by hypoxia and nitric oxide. J Biol Chem. 276(3): 2292-8.

Kivirikko KI, Myllyharju J (1998). Prolyl 4-hydroxylases and their protein disulfide isomerase subunit. Matrix Biol. 16(7): 357-68.

Knowles HJ, Raval RR, Harris AL, Ratcliffe PJ (2003). Effect of ascorbate on the activity of hypoxia-inducible factor in cancer cells. Cancer Res 63(8): 1764-8.

Koivunen P, Tiainen P, Hyvarinen J, Williams KE, Sormunen R, Klaus SJ, Kivirikko KI, Myllyharju J (2007). An endoplasmic reticulum transmembrane prolyl 4-hydroxylase is induced by hypoxia and acts on hypoxia-inducible factor alpha. J Biol Chem. 282(42): 30544-52.

Kondo Y, Inai Y, Sato Y, Handa S, Kubo S, Shimokado K, Goto S, Nishikimi M, Maruyama N, Ishigami A (2006). Senescence marker protein 30 functions as

gluconolactonase in L-ascorbic acid biosynthesis, and its knockout mice are prone to scurvy. Proc Natl Acad Sci U S A. 103(15): 5723-8.

Kuiper C, Molenaar IG, Dachs GU, Currie MJ, Sykes PH, Vissers MC (2010). Low Ascorbate Levels Are Associated with Increased Hypoxia-Inducible Factor-1 Activity and an Aggressive Tumor Phenotype in Endometrial Cancer. Cancer Res 2010: 22.

Laughner E, Taghavi P, Chiles K, Mahon PC, Semenza GL (2001). HER2 (neu) signaling increases the rate of hypoxia-inducible factor 1alpha (HIF-1alpha) synthesis: novel mechanism for HIF-1-mediated vascular endothelial growth factor expression. Mol Cell Biol. 21(12): 3995-4004.

Levine M, Espey MG, Chen Q (2009). Losing and finding a way at C: new promise for pharmacologic ascorbate in cancer treatment. Free Radic Biol Med. 47(1): 27-9.

Li SH, Ryu JH, Park SE, Cho YS, Park JW, Lee WJ, Chun YS (2009). Vitamin C supplementation prevents testosterone-induced hyperplasia of rat prostate by down-regulating HIF-1alpha. J Nutr Biochem 21(9): 801-8.

Li Y, Schellhorn HE (2007). New developments and novel therapeutic perspectives for vitamin C. J Nutr. 137(10): 2171-84.

Lim JH, Lee YM, Chun YS, Chen J, Kim JE, Park JW (2010). Sirtuin 1 modulates cellular responses to hypoxia by deacetylating hypoxia-inducible factor 1alpha. Mol 38(6): 864-78.

Lind J (1753). A Treatise of the Scurvy in Three Parts. Containing an Inquiry into the Nature, Causes and Cure of that Disease, together with a Critical and Chronological View of what has been published on the subject.

Linster CL, Van Schaftingen E (2007). Vitamin C. Biosynthesis, recycling and degradation in mammals. Febs J. 274(1): 1-22.

Mabjeesh NJ, Willard MT, Frederickson CE, Zhong H, Simons JW (2003). Androgens stimulate hypoxia-inducible factor 1 activation via autocrine loop of tyrosine kinase receptor/phosphatidylinositol 3'-kinase/protein kinase B in prostate cancer cells. Clin Cancer Res. 9(7): 2416-25.

Maeda N, Hagihara H, Nakata Y, Hiller S, Wilder J, Reddick R (2000). Aortic wall damage in mice unable to synthesize ascorbic acid. Proc Natl Acad Sci USA 97(2): 841-6.

Mandl J, Szarka A, Banhegyi G (2009). Vitamin C: update on physiology and pharmacology. Br J Pharmacol. 157(7): 1097-110.

Martensson J, Han J, Griffith OW, Meister A (1993). Glutathione ester delays the onset of scurvy in ascorbate-deficient guinea pigs. Proc Natl Acad Sci USA 90(1): 317-21.

Martin F, Linden T, Katschinski DM, Oehme F, Flamme I, Mukhopadhyay CK, Eckhardt K, Troger J, Barth S, Camenisch G, Wenger RH (2005). Copper-dependent activation of hypoxia-inducible factor (HIF)-1: implications for ceruloplasmin regulation. Blood. 105(12): 4613-9.

Martini E (2002). Jacques Cartier witnesses a treatment for scurvy. Vesalius. 8(1): 2-6.

Massip L, Garand C, Paquet ER, Cogger VC, O'Reilly JN, Tworek L, Hatherell A, Taylor CG, Thorin E, Zahradka P, Le Couteur DG, Lebel M (2009). Vitamin C restores healthy aging in a mouse model for Werner syndrome. Faseb J 24(1): 158-72.

Masson N, Appelhoff RJ, Tuckerman JR, Tian YM, Demol H, Puype M, Vandekerckhove J, Ratcliffe PJ, Pugh CW (2004). The HIF prolyl hydroxylase PHD3 is a potential substrate of the TRiC chaperonin. FEBS Lett. 570(1-3): 166-70.

McDonough MA, Li V, Flashman E, Chowdhury R, Mohr C, Lienard BM, Zondlo J, Oldham NJ, Clifton IJ, Lewis J, McNeill LA, Kurzeja RJ, Hewitson KS, Yang E, Jordan S, Syed RS, Schofield CJ (2006). Cellular oxygen sensing: Crystal structure of hypoxia-inducible factor prolyl hydroxylase (PHD2). Proc Natl Acad Sci U S A. 103(26): 9814-9.

Melillo G, Taylor LS, Brooks A, Musso T, Cox GW, Varesio L (1997). Functional requirement of the hypoxia-responsive element in the activation of the inducible nitric oxide synthase promoter by the iron chelator desferrioxamine. J Biol Chem. 272(18): 12236-43.

Metzen E, Berchner-Pfannschmidt U, Stengel P, Marxsen JH, Stolze I, Klinger M, Huang WQ, Wotzlaw C, Hellwig-Burgel T, Jelkmann W, Acker H, Fandrey J (2003). Intracellular localisation of human HIF-1 alpha hydroxylases: implications for oxygen sensing. J Cell Sci. 116(Pt 7): 1319-26.

Moertel CG, Fleming TR, Creagan ET, Rubin J, O'Connell MJ, Ames MM (1985). High-dose vitamin C versus placebo in the treatment of patients with advanced cancer who have had no prior chemotherapy. A randomized double-blind comparison. N Engl J Med. 312(3): 137-41.

Muellner MK, Schreier SM, Schmidbauer B, Moser M, Quehenberger P, Kapiotis S, Goldenberg H, Laggner H (2010). Vitamin C inhibits NO-induced stabilization of HIF-1alpha in HUVECs. Free 44(7): 783-91.

Myllyharju J (2008). Prolyl 4-hydroxylases, key enzymes in the synthesis of collagens and regulation of the response to hypoxia, and their roles as treatment targets. Ann Med 23: 1-16.

Nagel S, Talbot NP, Mecinovic J, Smith TG, Buchan AM, Schofield CJ (2010). Therapeutic manipulation of the HIF hydroxylases. Antioxid 12(4): 481-501.

Nakayama K, Gazdoiu S, Abraham R, Pan ZQ, Ronai Z (2007). Hypoxia-induced assembly of prolyl hydroxylase PHD3 into complexes: implications for its activity and susceptibility for degradation by the E3 ligase Siah2. Biochem J. 401(1): 217-26.

Nishikimi M, Koshizaka T, Ozawa T, Yagi K (1988). Occurrence in humans and guinea pigs of the gene related to their missing enzyme L-gulono-gamma-lactone oxidase. Arch Biochem Biophys. 267(2): 842-6.

Nytko KJ, Spielmann P, Camenisch G, Wenger RH, Stiehl DP (2007). Regulated function of the prolyl-4-hydroxylase domain (PHD) oxygen sensor proteins. Antioxid Redox Signal 9(9): 1329-38.

Oehme F, Ellinghaus P, Kolkhof P, Smith TJ, Ramakrishnan S, Hutter J, Schramm M, Flamme I (2002). Overexpression of PH-4, a novel putative proline 4-hydroxylase, modulates activity of hypoxia-inducible transcription factors. Biochem Biophys Res Commun. 296(2): 343-9.

Page EL, Chan DA, Giaccia AJ, Levine M, Richard DE (2008). Hypoxia-inducible factor-1alpha stabilization in nonhypoxic conditions: role of oxidation and intracellular ascorbate depletion. Mol Biol Cell. 19(1): 86-94.

Palmer LA, Semenza GL, Stoler MH, Johns RA (1998). Hypoxia induces type II NOS gene expression in pulmonary artery endothelial cells via HIF-1. Am J Physiol. 274(2 Pt 1): L212-9.

Park YK, Ahn DR, Oh M, Lee T, Yang EG, Son M, Park H (2008). Nitric oxide donor, (+/-)-S-nitroso-N-acetylpenicillamine, stabilizes transactive hypoxia-inducible factor-1alpha by inhibiting von Hippel-Lindau recruitment and asparagine hydroxylation. Mol Pharmacol. 74(1): 236-45.

Parsons KK, Maeda N, Yamauchi M, Banes AJ, Koller BH (2006). Ascorbic acid-independent synthesis of collagen in mice. Am J Physiol Endocrinol Metab 290(6): E1131-9.

Pauling L (1971). The significance of the evidence about ascorbic acid and the common cold. Proc Natl Acad Sci U S A. 68(11): 2678-81.

Pauling L (1977). Vitamin homeostasis in the brain and megavitamin therapy. N Engl J Med. 297(14): 790-1.

Qiao H, Li L, Qu ZC, May JM (2009). Cobalt-induced oxidant stress in cultured endothelial cells: prevention by ascorbate in relation to HIF-1alpha. Biofactors. 35(3): 306-13.

Richard DE, Berra E, Gothie E, Roux D, Pouyssegur J (1999). p42/p44 mitogen-activated protein kinases phosphorylate hypoxia-inducible factor 1alpha (HIF-1alpha) and enhance the transcriptional activity of HIF-1. J Biol Chem. 274(46): 32631-7.

Rius J, Guma M, Schachtrup C, Akassoglou K, Zinkernagel AS, Nizet V, Johnson RS, Haddad GG, Karin M (2008). NF-kappaB links innate immunity to the hypoxic response through transcriptional regulation of HIF-1alpha. Nature. 453(7196): 807-11.

Rockenfeller P, Madeo F (2010). Ageing and eating. Biochim Biophys Acta 1803(4): 499-506.

Sablina AA, Budanov AV, Ilyinskaya GV, Agapova LS, Kravchenko JE, Chumakov PM (2005). The antioxidant function of the p53 tumor suppressor. Nat Med. 11(12): 1306-13.

Salnikow K, Donald SP, Bruick RK, Zhitkovich A, Phang JM, Kasprzak KS (2004). Depletion of intracellular ascorbate by the carcinogenic metals nickel and cobalt results in the induction of hypoxic stress. J Biol Chem 279(39): 40337-44.

Salnikow K, Kasprzak KS (2005). Ascorbate depletion: a critical step in nickel carcinogenesis? Environ Health Perspect 113(5): 577-84.

Sang N, Stiehl DP, Bohensky J, Leshchinsky I, Srinivas V, Caro J (2003). MAPK signaling up-regulates the activity of hypoxia-inducible factors by its effects on p300. J Biol Chem. 278(16): 14013-9.

Sasabe E, Yang Z, Ohno S, Yamamoto T (2010). Reactive oxygen species produced by the knockdown of manganese-superoxide dismutase up-regulate hypoxia-inducible factor-1alpha expression in oral squamous cell carcinoma cells. Free Radic Biol Med 48(10): 1321-9.

Schafer M, Werner S (2008). Oxidative stress in normal and impaired wound repair. Pharmacol Res. 58(2): 165-71.

Scheid A, Wenger RH, Christina H, Camenisch I, Ferenc A, Stauffer UG, Gassmann M, Meuli M (2000). Hypoxia-regulated gene expression in fetal wound regeneration and adult wound repair. Pediatr Surg Int. 16(4): 232-6.

Schreml S, Szeimies RM, Prantl L, Karrer S, Landthaler M, Babilas P (2010). Oxygen in acute and chronic wound healing. Br 163(2): 257-68.

Semenza G (2002). Signal transduction to hypoxia-inducible factor 1. Biochem Pharmacol. 64(5-6): 993-8.

Semenza GL (2010). Defining the role of hypoxia-inducible factor 1 in cancer biology and therapeutics. Oncogene 29(5): 625-34.

Shibata A, Nakagawa K, Sookwong P, Tsuduki T, Tomita S, Shirakawa H, Komai M, Miyazawa T (2008). Tocotrienol inhibits secretion of angiogenic factors from human colorectal adenocarcinoma cells by suppressing hypoxia-inducible factor-1alpha. J Nutr. 138(11): 2136-42.

Shoshani T, Faerman A, Mett I, Zelin E, Tenne T, Gorodin S, Moshel Y, Elbaz S, Budanov A, Chajut A, Kalinski H, Kamer I, Rozen A, Mor O, Keshet E, Leshkowitz D, Einat P, Skaliter R, Feinstein E (2002). Identification of a novel hypoxia-inducible factor 1-responsive gene, RTP801, involved in apoptosis. Mol Cell Biol. 22(7): 2283-93.

Sivridis E, Giatromanolaki A, Gatter KC, Harris AL, Koukourakis MI (2002). Association of hypoxia-inducible factors 1alpha and 2alpha with activated angiogenic pathways and prognosis in patients with endometrial carcinoma. Cancer. 95(5): 1055-63.

Stiehl DP, Jelkmann W, Wenger RH, Hellwig-Burgel T (2002). Normoxic induction of the hypoxia-inducible factor 1alpha by insulin and interleukin-1beta involves the phosphatidylinositol 3-kinase pathway. FEBS Lett 512(1-3): 157-62.

Stiehl DP, Wirthner R, Köditz J, Spielmann P, Camenisch G, Wenger RH (2006). Increased prolyl 4-hydroxylase domain proteins compensate for decreased oxygen levels. Evidence for an autoregulatory oxygen-sensing system. J Biol Chem 281(33): 23482-91.

Telang S, Clem AL, Eaton JW, Chesney J (2007). Depletion of ascorbic acid restricts angiogenesis and retards tumor growth in a mouse model. Neoplasia 9(1): 47-56.

Tissot van Patot MC, Serkova NJ, Haschke M, Kominsky DJ, Roach RC, Christians U, Henthorn TK, Honigman B (2009). Enhanced leukocyte HIF-1alpha and HIF-1 DNA binding in humans after rapid ascent to 4300 m. Free Radic Biol Med. 46(11): 1551-7.

Triantafyllou A, Mylonis I, Simos G, Bonanou S, Tsakalof A (2008). Flavonoids induce HIF-1alpha but impair its nuclear accumulation and activity. Free Radic Biol Med. 44(4): 657-70.

Tsukiyama F, Nakai Y, Yoshida M, Tokuhara T, Hirota K, Sakai A, Hayashi H, Katsumata T (2006). Gallate, the component of HIF-inducing catechins, inhibits HIF prolyl hydroxylase. Biochem Biophys Res Commun. 351(1): 234-9.

Vissers MC, Gunningham SP, Morrison MJ, Dachs GU, Currie MJ (2007). Modulation of hypoxia-inducible factor-1 alpha in cultured primary cells by intracellular ascorbate. Free Radic Biol Med. 42(6): 765-72.

Vissers MC, Wilkie RP (2007). Ascorbate deficiency results in impaired neutrophil apoptosis and clearance and is associated with up-regulation of hypoxia-inducible factor 1α. J Leukoc Biol 81(5): 1236-44.

Welsh SJ, Bellamy WT, Briehl MM, Powis G (2002). The redox protein thioredoxin-1 (Trx-1) increases hypoxia-inducible factor 1alpha protein expression: Trx-1 overexpression results in increased vascular endothelial growth factor production and enhanced tumor angiogenesis. Cancer Res. 62(17): 5089-95.

Wenger RH, Camenisch G, Stiehl DP, Katschinski DM (2009). HIF prolyl-4-hydroxylase interacting proteins: consequences for drug targeting. Curr Pharm Des. 15(33): 3886-94.

Wingrove JA, O'Farrell PH (1999). Nitric oxide contributes to behavioral, cellular, and developmental responses to low oxygen in Drosophila. Cell. 98(1): 105-14.

Wirthner R, Balamurugan K, Stiehl DP, Barth S, Spielmann P, Oehme F, Flamme I, Katschinski DM, Wenger RH, Camenisch G (2007). Determination and modulation of prolyl-4-hydroxylase domain oxygen sensor activity. Methods Enzymol 435: 43-60.

Wolf J, Levy IJ (1954). Treatment of sickle cell anemia with cobalt chloride. AMA Arch Intern Med. 93(3): 387-96.

Yee Koh M, Spivak-Kroizman TR, Powis G (2008). HIF-1 regulation: not so easy come, easy go. Trends Biochem Sci. 33(11): 526-34.

Yuan Y, Hilliard G, Ferguson T, Millhorn DE (2003). Cobalt inhibits the interaction between hypoxia-inducible factor-alpha and von Hippel-Lindau protein by direct binding to hypoxia-inducible factor-alpha. J Biol Chem. 278(18): 15911-6.

Zhong H, Chiles K, Feldser D, Laughner E, Hanrahan C, Georgescu MM, Simons JW, Semenza GL (2000). Modulation of hypoxia-inducible factor 1alpha expression by the epidermal growth factor/phosphatidylinositol 3-kinase/PTEN/AKT/FRAP pathway in human prostate cancer cells: implications for tumor angiogenesis and therapeutics. Cancer Res. 60(6): 1541-5.

Zhong L, D'Urso A, Toiber D, Sebastian C, Henry RE, Vadysirisack DD, Guimaraes A, Marinelli B, Wikstrom JD, Nir T, Clish CB, Vaitheesvaran B, Iliopoulos O, Kurland I, Dor Y, Weissleder R, Shirihai OS, Ellisen LW, Espinosa JM, Mostoslavsky R (2010). The histone deacetylase Sirt6 regulates glucose homeostasis via Hif1alpha. Cell. 140(2): 280-93.

Zhou J, Damdimopoulos AE, Spyrou G, Brune B (2007). Thioredoxin 1 and thioredoxin 2 have opposed regulatory functions on hypoxia-inducible factor-1alpha. J Biol Chem. 282(10): 7482-90.

9. Unpublished Manuscript: Generation of monoclonal antibodies raised against full length human PHD3

Katarzyna J. Nytko, Roland H. Wenger, and Daniel P. Stiehl

Department of Physiology and Zürich Center for Integrative Human Physiology
ZIHP, University of Zürich, CH-8057 Zürich, Switzerland;

Introduction

Oxygen sensing via HIF prolyl-4-hydroxylases (PHDs) is a mechanism playing an important role in many physiological and pathophysiological processes including adaptation to high attitude, ischemia or cancer. Under normal oxygen supply PHDs hydroxylate two distinct proline residues (P564 and P402 in human HIF-1 α) within the HIF-α molecule, which targets HIF-α protein for proteosomal degradation (Ivan et al., 2001; Jaakkola et al., 2001). PHD activity declines as a function of oxygen. Consequently, when oxygen concentration is reduced, HIF-α becomes stabilized, translocates to the nucleus, where it homodimerizes with HIF-β subunits and in complex activates the expression of target genes involved in cellular and systemic adaptation to hypoxia (Wenger et al., 2005). HIF prolyl-4-hydroxylase enzymes belong to a larger superfamily of 2-oxoglutarate and iron (II) dependent di-oxygenases (Kaelin and Ratcliffe, 2008). In humans, PHD enzymes are expressed as three isoforms (PHD1-3) with distinct genes, called *EGLN2, EGLN1* and *EGLN3*, respectively . These three isoforms differ in size, intracellular localization as well as tissue distribution (Hirsilä et al., 2003). PHD3/EGLN3/HPH1 is the smallest out of the three isoforms of HIF prolyl-4-hydroxylases with only 239 aminoacids (27.3 kDa) and two alternative splice variants (24 and 17 kDa) (Cervera et al., 2006). Expression of this isoform is particularly high in the heart (Stiehl et al., 2006). In contrast to the other two isoenzymes, PHD3 hydroxylates preferentially P564 and has little activity on P402 located in the C-terminal part of HIF-1α. Moreover, a tendency of PHD3 towards the hydroxylation of HIF-2α has been reported (Chan et al., 2005; Katschinski, 2009; Villar et al., 2007). In line with these findings derived from cell culture experiments, mice with targeted disruption of both Phd3 allels have abnormal sympathoadrenal development, and intercrossing PHD3(-/-) mice with HIF-1α and

HIF-2α(+/-) heterozygous mice demonstrated an interaction with only HIF-2α (Bishop et al., 2008). This phenotype might be caused by imbalanced cell survival of neuronal cells, since PHD3 was previously shown to play an important role in neuronal apoptosis (Rantanen et al., 2008).

Interestingly, the genes encoding PHD2 and PHD3 are regulated by hypoxia in a HIF-dependent manner and functional hypoxia response elements (HRE) have been identified in their promoter and first intronic regions, respectively (Metzen et al., 2005; Pescador et al., 2005) . Thus, the oxygen sensing HIF/PHD system is coupled by a negative feedback loop, allowing cells to adapt to chronic hypoxia by decreasing HIF levels at least partially by increased expression of PHD2 and PHD3 enzymes (Minamishima et al., 2009; Stiehl et al., 2006).

Understanding the molecular pathways, which underlies effects of PHD3 and HIF proteins is a milestone in developing potential treatment of hypoxia-related diseases. Therefore, monoclonal antibodies against PHDs are important tools to study their cellular and tissue expression pattern as well as involvement in number of physiological and pathophysiological processes. Antibodies against PHD1-3 are comercially available on the market, however, the quality and application of these antibodies is not always sufficient and depends on many factors, such as the cell line or technique used. Due to the lack of well working anti-PHD3 antibodies we attempted to generate monoclonal anti- PHD3 antibodies in mice.

Material and methods

Antigen generation and immunization of mice

Full length GST-tagged PHD3 was expressed in Sf9 and purified as described before (Stiehl et al., 2006). Briefly, after 110 hours of infection Sf9 cells were lysed in ice-cold 0.1% NP-40, 10 mM Tris-HCl pH 7.5, 100 mM NaCl, 100 mM glycine and 10 µM DTT. Cleared lysates were incubated with equilibrated glutathione-sepharose beads (Amersham, Dübendorf, Switzerland) for 2 hours at 4°C with gentle agitation. After washing of the bead bed for three times with PBS, bound protein was eluted with 15 mM reduced glutathione, 50 mM Tris-HCl pH 8.0, and 2 µM $FeSO_4$. Purity of recombinant fusion-proteins was routinely estimated by SDS-PAGE and coomassie blue staining. Immunization of mice and generation of hybridoma fusion was performed in the laboratory of Dr. Rene Fischer (ETH Zürich).

Expression and purification of MBP.PHD3 for screening of hybridoma supernatants by ELISA

Full-length MBP-tagged PHD3 was expressed in *E. Coli* TB.1 cells and purified using amylose resins. Briefly, 1L of o/n bacterial culture in the logarithmic growth phase (O.D at 600 nm between 0.4-0.6) was induced with 300 µM IPTG for 4 hours. Cells were collected, resuspended in 20 ml of lysis buffer (20 mM Tris/Cl, pH 7.4, 200 mM NaCl and 1 mM EDTA) and lysed at 2.7 kbar using a French press. Cleared lysate was incubated with equilibrated amylose resins for 2 hours at 4°C with gentle agitation. After washing three times with lysis buffer, bound protein was eluted with 10 mM D-maltose in lysis buffer. Purity of the recombinant fusion-protein was routinely estimated by SDS-PAGE and coomassie blue staining.

Subcloning of hybridoma clones

Obtained fusion hybridoma cells were plated on a 96-well plate in chemically defined Turbudoma medium (www.cellculture.com) containing gentamycin (50 µg/ml), L-glutamine (2 mM), Interleukin-6 (2%; vol/vol) and 1×hypoxanthine-aminopterin-thymidine medium (HAT). 6.5×10^7 cells were plated on six 96-well plates (200µl/well). Hybridoma cultures were grown for 7-10 days and positive wells were visually screened for single cell clusters, before supernatants of the clones were tested

by ELISA. Positive clones were further subcloned by limited dilution on 96-well plates and subsequently tested by ELISA and immunoblotting.

Cell culture for production of monoclonal antibodies

Hybridoma clones were routinely maintained in Turbudoma (www.cellculture.com) medium and the supernatants were collected for analysis and purification. For high-density antibody production, cells were grown in a BD CELLine 1000 flask. 2×10^6 cells/ml were resuspended in 15 ml of Turbodoma medium and inoculated into the cell compartment of the BD CELLine flask. The nutrient compartment was filled with 1L of Turbudoma medium and cells were grown for 7-14 days. Afterwards cells were collected from the cell compartment and the supernatant containing concentrated antibodies was tested by ELISA and immunoblotting. Antibodies were isotyped using a Mouse Monoclonal Antibody Isotyping Kit (Roche).

ELISA

To select for positive hybridoma supernatants, 96-well plates were coated with 3 µg/ml (100µl/well) of either recombinant GST.PHD3 purified from infected Sf9 cells or bacterial recombinant MBP-PHD3 fusion protein. The coating was performed for 2h at 37 ºC with protein diluted in PBS. To avoid unspecific binding, plates were blocked with blocking buffer containing 2% non-fat dry milk (weight/vol), 150 mM NaCl in 20 mM Tris buffer, pH 7.4. Subsequently, wells were washed five times with washing buffer containing 0.05% Tween 20 and 150 mM NaCl in 20 mM Tris, pH 7.4. Hybridoma supernatants (100µl/well) were incubated for 3 hours at room temperature and afterwards plates were washed five times with washing buffer. Secondary HRP-conjugated anti-mouse antibodies (Pierce, dilution 1:1000) was added to each well and plates were incubated for 2 hours at room temperature followed by five washing steps. For detection, the 3,3',5,5'-tetramethylbenzidine (TMB) substrate kit (Pierce) was used and blue color development was stopped by adding H_2SO_4 to 1 M. Absorbance was determined at 450 nm in a microplate reader.

Purification of IgG molecules

Supernatants of hybridoma clones were pooled and loaded with 0.5 – 0.8 ml/min on an equilibrated (PBS) Protein G column (HiTrap) Afterwards the column was washed with PBS and antibodies were stepwise eluted with elution buffer (100 mM Glycine,

150 mM NaCl) at pH 2.6 and 2.2, respectively. Eluted fractions were immediately neutralized by addition of 1M Tris/Cl pH 8.0. Flow through and eluted fractions were analyzed by SDS-PAGE.

Immunoblotting

For a routine screen of hybridoma supernatants, 20ng/lane of recombinant GST or MBP-tagged PHD were subjected to SDS-PAGE and electrotransferred onto PVDF membranes. Subsequently, membranes were incubated with undiluted hybridoma supernatants or purified antibodies for at least 2h at room temperature. Secondary HRP-conjugated anti-mouse antibodies were used for detection. Endogenous and overexpressed PHD3 protein was detected in whole cell lysates of MCF-7 or HEK293 cells transfected with 4 μg of pcDNA3.1-hPHD3.

Immunohistochemistry

Staining of human lung adenocarcinoma with supernatant of hybridoma subclone A1.10 was performed in cooperation with Dr. Peter Schraml (Clinical Pathology/University Hospital Zürich).

Results and discussion

Recombinant GST.PHD3 protein purified from Sf9 insect cells was used for immunization of mice as described previously (Figure 1A) (Baggio et al., 2005). Supernatants from the obtained hybridoma fusion cells were tested for the reactivity with recombinant GST.PHD3 proteins. Several clones were positive for detection of recombinant antigen (Figure 1B) however, they were also positive for detection of GST protein, which was the fusion partner of PHD3 antigen used for immunization, (lower band, around 25 kDa (Figure 1A and C). Additionally, Sf9 lysates overexpressing His_6–PHD3 were not detected by clone A5 supernatant, which readily detected GST-PHD3 (Figure 1B and C). In order to avoid false-positive signals, bacterially expressed MBP.PHD3 was purified and used for subsequent ELISA and immunoblot analyses (Figure 1D). Several clones were positive for anti-MBP.PHD3 detection by ELISA, with clone A1 having the highest absorbance relative to the commercially available polyclonal antibody NB-100-303 (Novus Biologies) (Figure 1E). Note, that affinity-purified NB-100-303 was used at a final concentration of 1 ug/ml, which supposedly was much higher than the concentration of respective hybridoma supernatants. All positive clones were further tested by immunoblotting and clones A1, B3 and B4 were further subcloned.

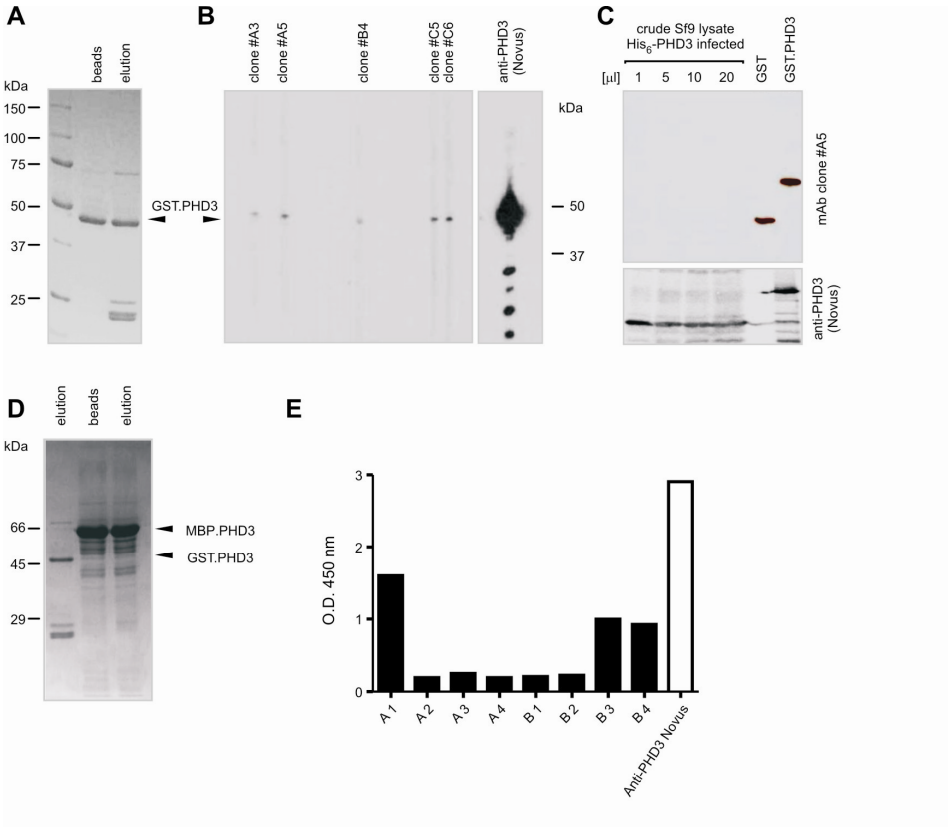

Fig. 1. Generation of monoclonal antibodies against PHD3. (A) Purification of recombinant GST-PHD3 from Sf9 insect cells. Note the presence of the band at around 25 kDa corresponding to GST alone. (B) Testing hybridoma supernatants on recombinant GST-PHD3 by immunobloting using a multiple sera applicator. (C) Supernatant from hybridoma clone A5 detects recombinant GST protein and GST-PHD3, but fails to detect His_6-tagged PHD3 in transfected Sf9 cell lysates (upper panel). The commercially available anti-PHD3 antibodies were used as a control to detect His_6-tagged as well as GST-PHD3 (lower panel). (D) Generation of bacterially purified MBP.PHD3. (E) ELISA using supernatants from hybridoma clones on MBP.PHD3 with clone A1 showing highest absorbance.

Supernatant from clone A1 was further collected for small-scale purification as shown in Figure 2. Binding of the heavy chains of the immunoglobulin to either Protein A or G greatly depends on the species and isotype of the antibodies (Harlow Ed, 1999). Accordingly, clone A1 was isotyped as IgG1 kappa and had higher affinity for protein G resins (Figure 2A). For purification of the antibodies from the beads, elution buffers with pH 2.7 and pH 2.2 were used. Antibodies were found to elute from the column more efficiently at lower pH (Figure 2B). Although elution with buffer pH 2.7 gave higher absorbances at the chromatogram, antibodies were found at higher concentration in the elution buffer at pH 2.2 (Figure 2B and C). After purification, fraction 32 and flow through were tested to detect recombinant MBP.PHD3 by immunoblotting. Immunoreactivity against MBP.PHD3 of fraction 32 was titered up to dilutions of 1:1000, with a titer of 1:100 giving similar results as undiluted cell culture supernatant of A1 hybridoma cells (Figure 2D).

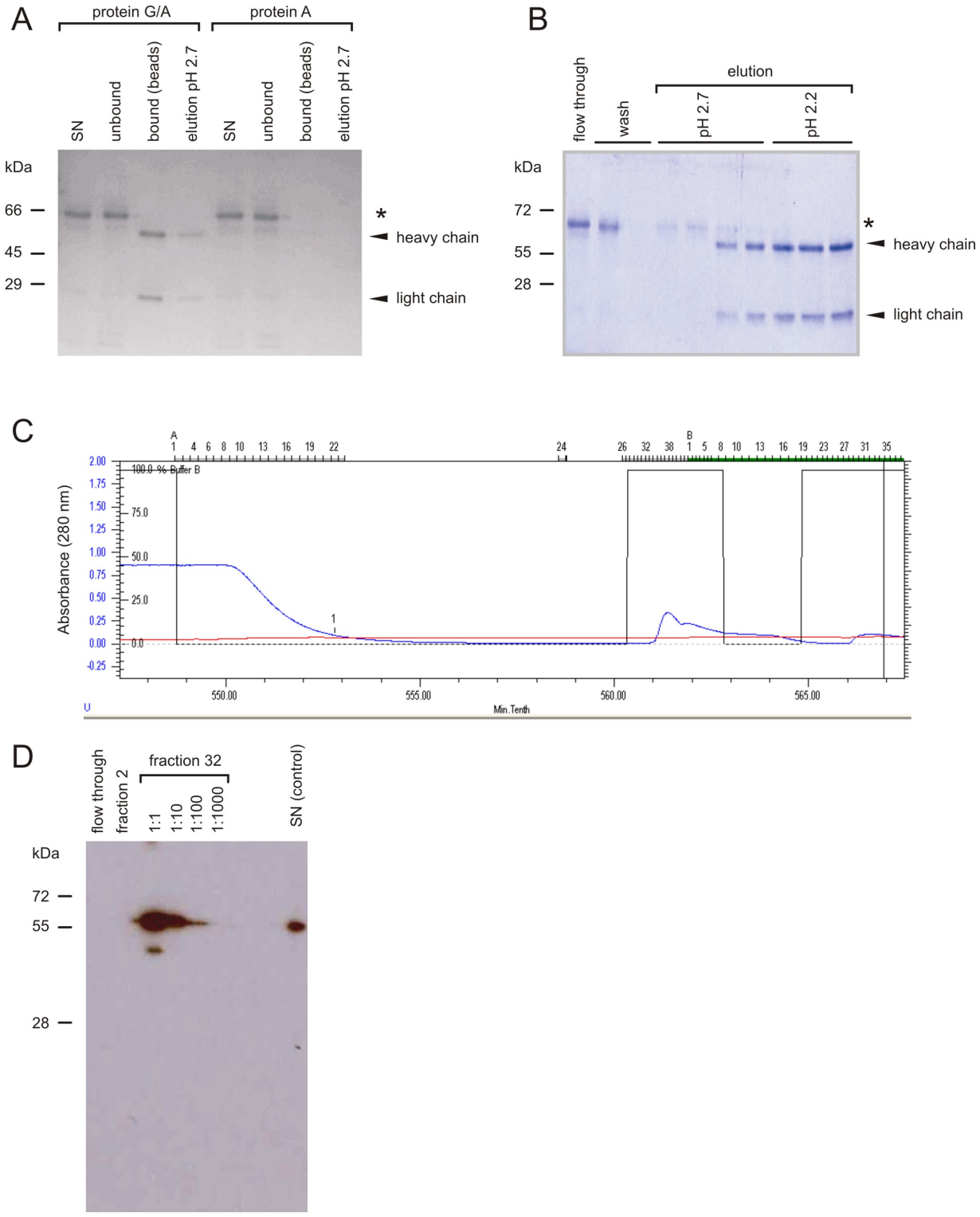

Fig 2. Purification of monoclonal anti-PHD3 antibodies from A1 hybridoma supernatant. (A) Antibodies from hybridoma supernatants of clone A1 preferentially bind to Protein G. (B) SDS-PAGE of eluted fraction containing anti-PHD3 immunoglobulin. (C) Chromatogram showing the elution profile of immunoglobulins with decreasing pH. (D) Titer analyses of eluted anti-PHD3 antibodies (fraction 32) on recombinant PHD3. Asterisk indicates protein present in the cell culture medium.

Further subcloning of the clone A1 resulted in a single subclone named A1.10, which had a high signal (O.D.450 nm = 1.255) in ELISA for detection of MBP.PHD3, which was roughly two-fold higher than the polyclonal control antibody NB-100-303 (1 ug/ml) with an O.D. at 450 nm of 0.5955. Using N-terminally truncated PHD3 protein exogenously expressed in HEK293 cells, we observed that purified A1.10 antibodies preferentially detect the full-length protein, while all PHD truncations were readily expressed as determined by V5 immunoblotting (Figure 3A). Moreover A1.10 at a dilution of 1:50 detected endogenous PHD3 protein in lysates of DMOG and hypoxia treated MCF-7 cells, though strikingly high amounts (400 µg/lane) of total protein were required (Figure 3B).

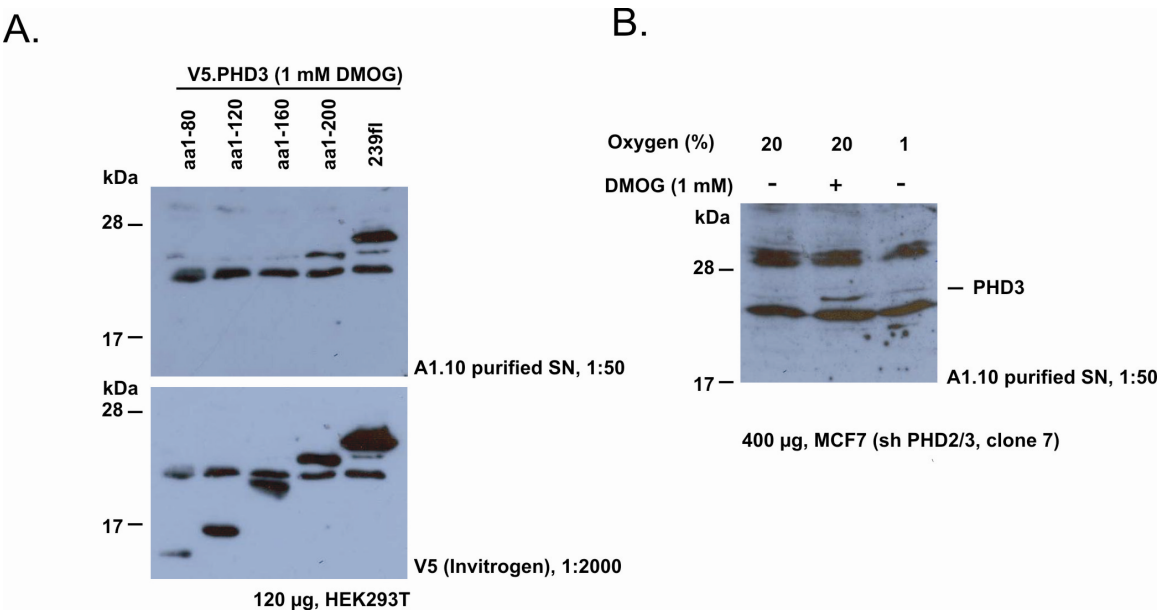

Fig. 3. Testing of supernatants from A1.10 for detection of overexpressed PHD3 in HEK293 cells. (A) Immunoblot of truncated V5.PHD3 constructs exogenously expressed in HEK293 cells treated with 1 mM DMOG (upper panel). Immunoblot using anti-V5 antibodies served as control (lower panel). (B) Detection of endogenous PHD3 cells in lysates of MCF7 cells treated with 1 mM DMOG or 1% oxygen.

When tested in immunohistochemistry of human tissues assembled in tissue microarrays, subclone A1.10 was staining several lung adenocarcionoma paraffin sections of different patients (Figure 4A). If compared with the performance of the monoclonal antibodies A1 and A1.10 in immunoblotting, these results might suggest a preference of both immunoglobulins to native PHD3. As such, the antibodies could have an application for immunohistochemistry or presumably in immunofluorescence.

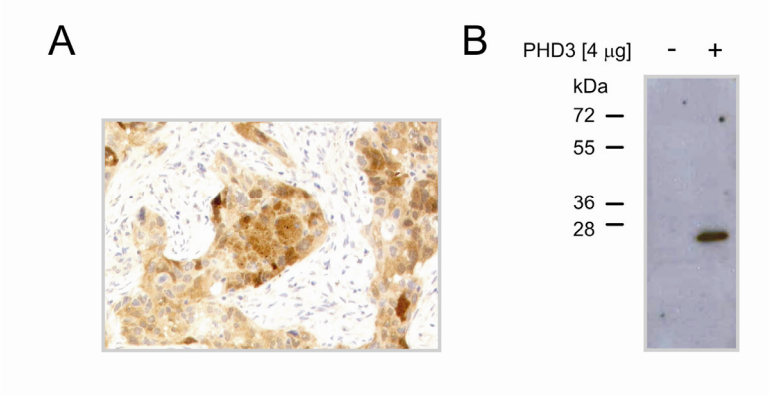

Fig. 4. Application of anti-PHD3 antibodies in immunohistochemistry and immunoblotting of exogenous PHD3. (A) Paraffin sections of human adenocarcinoma of the lung stained with supernatant of subclone A1.10. (B) Immunoblot of PHD3 overexpressed in MCF7 cells tested with supernatant of subclone A1.10.

References

Baggio V, Ott F, Fischer RW, Gram H, Peele J, Spreng D, Schmokel H, Jungi TW (2005). Production of antibodies to canine IL-1beta and canine TNF to assess the role of proinflammatory cytokines. Vet Immunol Immunopathol. 107(1-2): 27-39.

Bishop T, Gallagher D, Pascual A, Lygate CA, de Bono JP, Nicholls LG, Ortega-Saenz P, Oster H, Wijeyekoon B, Sutherland AI, Grosfeld A, Aragones J, Schneider M, van Geyte K, Teixeira D, Diez-Juan A, Lopez-Barneo J, Channon KM, Maxwell PH, Pugh CW, Davies AM, Carmeliet P, Ratcliffe PJ (2008). Abnormal sympathoadrenal development and systemic hypotension in PHD3-/- mice. Mol Cell Biol. 28(10): 3386-400.

Cervera AM, Apostolova N, Luna-Crespo F, Sanjuan-Pla A, Garcia-Bou R, McCreath KJ (2006). An alternatively spliced transcript of the PHD3 gene retains prolyl hydroxylase activity. Cancer Lett. 233(1): 131-8.

Chan DA, Sutphin PD, Yen SE, Giaccia AJ (2005). Coordinate regulation of the oxygen-dependent degradation domains of hypoxia-inducible factor 1 alpha. Mol Cell Biol. 25(15): 6415-26.

Harlow Ed DL (1999). Using Antibodies. New York, Cold Spring Harbor Laboratory Press.

Hirsilä M, Koivunen P, Günzler V, Kivirikko KI, Myllyharju J (2003). Characterization of the human prolyl 4-hydroxylases that modify the hypoxia-inducible factor. J Biol Chem 278(33): 30772-80.

Ivan M, Kondo K, Yang H, Kim W, Valiando J, Ohh M, Salic A, Asara JM, Lane WS, Kaelin WG, Jr. (2001). HIFα targeted for VHL-mediated destruction by proline hydroxylation: implications for O2 sensing. Science 292(5516): 464-8.

Jaakkola P, Mole DR, Tian YM, Wilson MI, Gielbert J, Gaskell SJ, Kriegsheim A, Hebestreit HF, Mukherji M, Schofield CJ, Maxwell PH, Pugh CW, Ratcliffe PJ (2001). Targeting of HIF-α to the von Hippel-Lindau ubiquitylation complex by O2-regulated prolyl hydroxylation. Science 292(5516): 468-72.

Kaelin WG, Jr., Ratcliffe PJ (2008). Oxygen sensing by metazoans: the central role of the HIF hydroxylase pathway. Mol Cell. 30(4): 393-402.

Katschinski DM (2009). In vivo functions of the prolyl-4-hydroxylase domain oxygen sensors: direct route to the treatment of anaemia and the protection of ischaemic tissues. Acta Physiol (Oxf). 195(4): 407-14.

Metzen E, Stiehl DP, Doege K, Marxsen JH, Hellwig-Burgel T, Jelkmann W (2005). Regulation of the prolyl hydroxylase domain protein 2 (phd2/egln-1) gene: identification of a functional hypoxia-responsive element. Biochem J. 387(Pt 3): 711-7.

Minamishima YA, Moslehi J, Padera RF, Bronson RT, Liao R, Kaelin WG, Jr. (2009). A feedback loop involving the Phd3 prolyl hydroxylase tunes the mammalian hypoxic response in vivo. Mol Cell Biol. 29(21): 5729-41.

Pescador N, Cuevas Y, Naranjo S, Alcaide M, Villar D, Landazuri MO, Del Peso L (2005). Identification of a functional hypoxia-responsive element that regulates the expression of the egl nine homologue 3 (egln3/phd3) gene. Biochem J. 390(Pt 1): 189-97.

Rantanen K, Pursiheimo J, Hogel H, Himanen V, Metzen E, Jaakkola PM (2008). Prolyl hydroxylase PHD3 activates oxygen-dependent protein aggregation. Mol Biol Cell. 19(5): 2231-40.

Stiehl DP, Wirthner R, Köditz J, Spielmann P, Camenisch G, Wenger RH (2006). Increased prolyl 4-hydroxylase domain proteins compensate for decreased oxygen levels. Evidence for an autoregulatory oxygen-sensing system. J Biol Chem 281(33): 23482-91.

Villar D, Vara-Vega A, Landazuri MO, Del Peso L (2007). Identification of a region on hypoxia-inducible-factor prolyl 4-hydroxylases that determines their specificity for the oxygen degradation domains. Biochem J. 408(2): 231-40.

Wenger RH, Stiehl DP, Camenisch G (2005). Integration of oxygen signaling at the consensus HRE. Sci STKE 2005(306): re12.

10. Unpublished data: Compounds tested in the *in vitro* hydroxylation assay for the effect on HIF prolyl-4-hydroxylases

Several compounds were tested for their effect on PHD1-3 hydroxylation activity using a VBC-dependent hydroxylation assay as described (Nytko et al., 2007). Briefly, antioxidants, reactive oxygen species, transition metals and metal chelators were added to the standard assay. Metal chelators, such as desferoxamine and ciclopirox olamine are commonly used to stabilize HIF-1α protein levels via inhibition of PHDs (Linden et al., 2003; Melillo et al., 1997; Wang and Semenza, 1993; Wanner et al., 2000). We wanted to test, whether other metal chelators like EDTA or NTA could affect PHDs activity. Indeed, EDTA was inhibiting all three PHDs but only at high concentrations of this compound (>100 μM). Surprisingly, EDTA was slightly increasing the activity of all three PHDs at concentrations <10 μM probably due to chelation of some metals present in the reaction buffer, which could inhibit PHDs. NTA was not affecting or in the case of PHD3 slightly inhibiting activity of PHDs. Both EDTA and NTA chelate ions such as Ca (II) or Fe (III) with high affinity. However, NTA is known to be less stable in complex solutions which could potentially explain the differences observed by us. Of note, the high affinity iron chelator DFX was the most potent inhibitor of PHDs (Table 1).

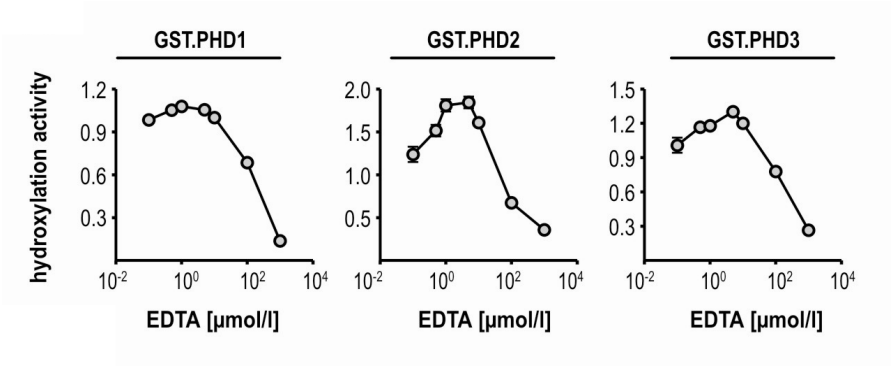

Fig. 1. Effect of EDTA on hydroxylation activity of PHD1-3 as tested by *in vitro* hydroxylation assay.

Besides metal chelators, also transition metals are potent inhibitors of PHDs hydroxylation activity (Hirsila et al., 2005). We tested cobalt (II) chloride (see Manuscript #2) and zinc (II) chloride (Figure 2). Both of them were efficient

inhibitors of all three PHDs, however PHD2 was more resistant to inhibition by both metals. Inhibition of PHDs by transition metals is a long-discussed effect but the mechanism is still not clear. It is suggested, that transition metals either replace iron (II) in the active center or cause oxidation of the enzyme or depletion of necessary co-factors (ascorbate).

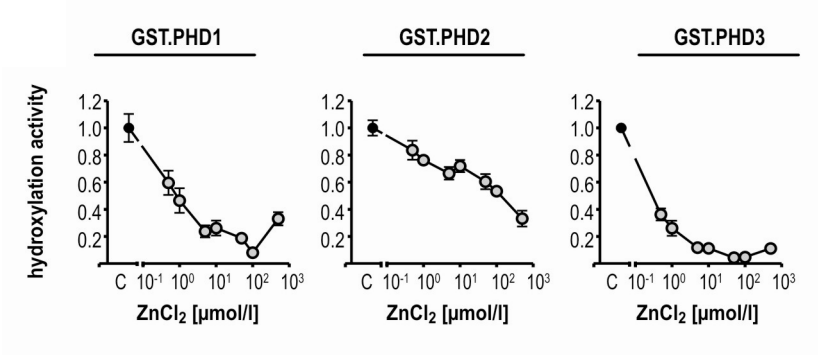

Fig. 2. Effect of zinc chloride on hydroxylation activity of PHD1-3 as tested by *in vitro* hydroxylation assay.

We were further testing effects of reactive oxygen species and antioxidants on hydroxylation activity of PHDs. As shown in manuscript #2, hydrogen peroxide was an efficient inhibitor of all three PHDs, though again rather high concentrations were needed for full inhibition of PHDs. We were interested though, if this effect could be reversed by addition of catalase, which is the main intracellular antioxidant responsible for dismutation of hydrogen peroxide to water and molecular oxygen. When enzymes were first incubated with hydrogen peroxide and subsequently with catalase before starting the hydroxylation reaction, the presence of catalase could reverse inhibitory effects of hydrogen peroxide (Figure 3). While this could be simply explained by detoxification of residual hydrogen peroxide by catalase, we could show that ROS modify the enzyme itself due to oxidative damage (manuscript #2). Whether such oxidative modifications (e.g. carbonylation) inhibit the specific activity of PHDs needs further investigation.

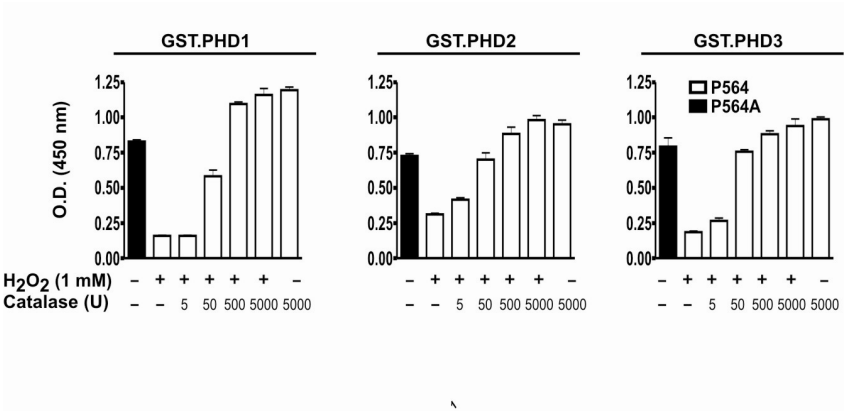

Fig. 3. Effect of hydrogen peroxide and catalase co-titration on PHD 1-3 as tested by *in vitro* hydroxylation assay.

In addition. sodium azide, which acts as inhibitor of complex IV (cytochrom oxidase) of the respiratory chain was tested in the VBC-coupled hydroxylation assay . Even at a concentration of 1 mM this compound had no influence on the activity of PHD1 and only slight reduced hydroxylation activities of PHD2 and PHD3 (Figure 4). Obviously, these data suggest that inhibitors of respiratory chain require a cellular system to exert effects on the oxygen sensing pathway via PHDs.

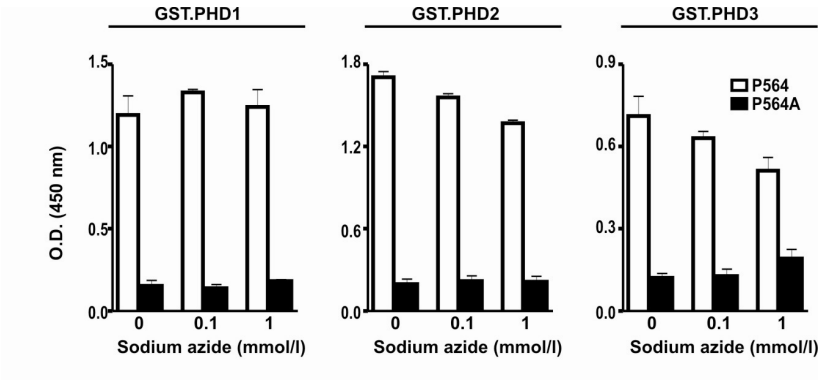

Fig. 4. Effect of sodium azide on hydroxylation activity of PHD 1-3 as tested by *in vitro* hydroxylation assay.

A summary of the different types of compounds tested for putative inhibitory or stimulatory effects on PHD mediated HIF-1α hydroxylation is shown in Table 1. As expected, most of the metal chelators, with exception for the zinc preselective chelator TPEN (*N*,*N*,*N*′,*N*′-tetrakis (2-pyridylmethyl) ethylenediamine) were efficient inhibitors of all three PHDs. It has been shown that TPEN can increase the activity of PHD2, however with our settings we did not observe similar result (Choi et al., 2006).

Other compounds with antioxidative properties such as the food additive gallate or the superoxide dismutase mimetic Mn (MnTMPyP) had inhibitory effects on PHD activity, suggesting different ways of action of specific antioxidants. Hydrogen peroxide was efficiently reducing hydroxylation activity which could be restored by subsequent addition of catalase. These novel data provide further evidence that antioxidants and ROS are important factors affecting the HIF pathway by directly modulating the hydroxylation capacity of the oxygen-sensing PHD enzymes.

Compound	Property	PHD1	PHD2	PHD3
DFX (0.1 – 1000 µM)	Chelating agent (Fe)	↓	↓	↓
EDTA (0.1 -1000 µM)	Chelating agent (Fe)	↓	↓	↓
TPEN (5 – 50 µM)	Chelating agent (Zn)	—	—	↓
Zn (0.5-500 µM)	Transition metal	↓	↓	↓
MnTMPyP (5 -500 µM)	Superoxide dismutase mimetic	↓	↓	↓
Gallic acid (3.3 -1000 µM)	Antioxidant	↓	↓	↓
Resveratrol (1 -330 µM)	Antioxidant	—	—	—
N-propyl-gallate (3.3 -1000 µM)	Antioxidant	↓	↓	↓
Succinate (0.01 -10mM)	Krebs cycle intermediate	↓	↓	↓
H_2O_2(0.004-2.7 mM)	ROS	↓	↓	↓
Sodium azide (0.1 -1mM)	Inhibitor of mitochondrial respiratory chain	—	—	—
Nitrilotriacetic acid NTA (0.001-10 mM)	Chelating agent (Fe)	—	—	—
Fludarabine (0.1 -10 µM)	Purine analog	—	—	—

Table 1. Compilation of small molecules inhibitors of PHD1-3 activity *in vitro*. The range of concentrations used for *in vitro* assays is given. Arrows indicate inhibitory properties of the respective compound.

References:

Choi SM, Choi KO, Lee N, Oh M, Park H (2006). The zinc chelator, N,N,N',N'-tetrakis (2-pyridylmethyl) ethylenediamine, increases the level of nonfunctional HIF-1alpha protein in normoxic cells. Biochem Biophys Res Commun. 343(4): 1002-8.

Hirsila M, Koivunen P, Xu L, Seeley T, Kivirikko KI, Myllyharju J (2005). Effect of desferrioxamine and metals on the hydroxylases in the oxygen sensing pathway. Faseb J. 19(10): 1308-10.

Linden T, Katschinski DM, Eckhardt K, Scheid A, Pagel H, Wenger RH (2003). The antimycotic ciclopirox olamine induces HIF-1alpha stability, VEGF expression, and angiogenesis. Faseb J. 17(6): 761-3.

Melillo G, Taylor LS, Brooks A, Musso T, Cox GW, Varesio L (1997). Functional requirement of the hypoxia-responsive element in the activation of the inducible nitric oxide synthase promoter by the iron chelator desferrioxamine. J Biol Chem. 272(18): 12236-43.

Nytko KJ, Spielmann P, Camenisch G, Wenger RH, Stiehl DP (2007). Regulated function of the prolyl-4-hydroxylase domain (PHD) oxygen sensor proteins. Antioxid Redox Signal 9(9): 1329-38.

Wang GL, Semenza GL (1993). Desferrioxamine induces erythropoietin gene expression and hypoxia-inducible factor 1 DNA-binding activity: implications for models of hypoxia signal transduction. Blood. 82(12): 3610-5.

Wanner RM, Spielmann P, Stroka DM, Camenisch G, Camenisch I, Scheid A, Houck DR, Bauer C, Gassmann M, Wenger RH (2000). Epolones induce erythropoietin expression via hypoxia-inducible factor-1 alpha activation. Blood. 96(4): 1558-65.

11. Conclusions

Oxygen sensing via the HIF-PHD pathway can be regulated at different levels and by different mechanisms. The primary factor regulating HIF protein level is oxygen, which drives the catalytic activity of HIF prolyl-4-hydroxylases (Epstein et al., 2001; Hirsilä et al., 2003). Besides oxygen, also the availability of co-factors as 2-oxoglutarate, iron (II) and to some extent ascorbate can influence the hydroxylation capacity of PHDs (Hirsilä et al., 2003). Consequently, compounds which cause depletion of one of the co-factors can moduluate the HIF response. Amongst them, the most effective PHD inhibitors are 2-oxoglutarate analogons (e.g. N-oxalylglycin), metal chelators (DFX, CPX and EDTA), transition metals (cobalt (II), zinc (II) , nickel (II) , copper (II)) and reactive oxygen species (H_2O_2, NO) (Hirsila et al., 2005; Salnikow et al., 2004; Martin et al., 2005 ; Page et al., 2008; Oehme et al., 2004). Similarly, some antioxidants (gallate and n-propyl gallate) as well as 2-oxoacids (succinate, fumarate, pyruvate) affect the activity of PHDs (Nytko et al., 2007; Pan et al., 2007). Of note, most of these compounds can inhibit (or induce) PHD activity even under oxic conditions and therefore modulate HIF hydroxylation independently of the oxygen concentration.

11.1 Role of ascorbate for PHD hydroxylation activity *in vitro*

Even though ascorbate is frequently described in the literature as essential co-factor for the activity of prolyl-4-hydroxylases, it remains speculative what the specific function of ascorbate in the reaction catalyzed by PHDs actually is (Hirsilä et al., 2003). We could show that besides ascorbate, also glutathione could induce hydroxylation activity of PHDs *in vitro*, even in the absence of ascorbate. While this work was in preparation, glutathione was shown to induce the activity of N-terminally truncated recombinant PHD2 at millimolar concentrations, which well reflects the intracellular glutathione content (Flashman et al., 2010). Moreover, glutathione was an efficient stimulator of FIH activity as shown by *in vitro* hydroxylation of an artificial consensus ankyrin peptide (Flashman et al., 2010). Glutathione, similarly to ascorbate could reduce the induction of a HIF-dependent luciferase reporter gene activity in hepatoma cells treated with the hypoxia-mimicking agent cobalt chloride.

Interestingly, neither ascorbate nor glutathione affected HIF activity induced by hypoxia or the iron chelator DFX (see Manuscript #2). Accordingly, glutathione could reduce carbonylation, which is the hallmark of protein oxidation, of recombinant PHD2 enzyme by Fenton reaction and cobalt chloride. It remains speculative, whether the function of ascorbate and glutathione on PHDs is mediated by the same mechanism. Ascorbate function in hydroxylation activity of PHDs is often suggested to maintain the active centre Fe (II) in the reduced state (Kaelin and Ratcliffe, 2008). The antioxidative function of glutathione in the cell is exerted mainly by serving as co-factor for glutathione peroxidases. Besides that, glutathione itself can scavenge a number of free radicals and other reactive compounds ($OH^{\bullet}$, HOCl etc.), with exception for the superoxide anion (Halliwell and Gutteridge, 2007). Glutathione is also a potent reducing agent, regulating the redox state of thiol groups in proteins. Two cysteine residues in PHD2 (Cys201 and Cys208) were described as surface accessible and highly nucelophilic aminoacids (Mecinovic et al., 2009). We generated recombinant Cys201Ser mutant PHD2 protein, which surprisingly showed a higher specific activity than the wildtype preparations of the PHD2 enzyme. However, glutathione could re-activate both wt and Cys201Ser mutant PHD2 enzymes after complete inactivation of the enzymes by hydrogen peroxide, suggesting that glutathione effects on PHD2 activity involves other mechanisms which are independent of Cys201 redox state. Additionally, glutathione plays an important role in the recycling of intracellular ascorbate. The latter mechanism might be excluded in the case of PHDs, as we *in vitro* observed an increase of PHD hydroxylation activity even in the absence of ascorbate. Still, there could have been undetectable traces of dehydroascorbate co-purified with the enzymes preparations, which are then reduced by glutathione. In cellular systems, these two antioxidants are tightly connected by redox-mediated mechanisms, therefore any change in the concentration of either one can affect the over-all redox state of the cell and thus PHD activity.

11.2 Role of ascorbate in oxygen sensing *in vivo*

Using HeLa cells grown in the absence of ascorbate we could show that the response to hypoxia in these cells is fully functional. To study the hypoxic responses *in vivo*, we used $Gulo^{-/-}$ mice, which are unable to synthesize ascorbate, therefore dependent on its supplementation in the diet (Maeda et al., 2000). Ascorbate deficiency is known

to cause scurvy, a disease resulting from insufficient hydroxylation of collagen building connective tissues (Mandl et al., 2009). We found no evidence, that ascorbate deficiency affects oxygen sensing via the PHD-HIF pathway in Gulo$^{-/-}$ mice. Ascorbate depleted animals showed no changes in the expression of HIF-target genes in a variety of tissues, even after 5 weeks of ascorbate withdrawal which has been shown to sufficiently exhaust intracellular ascorbate stores. Moreover, the systemic response of Gulo$^{-/-}$ mice kept on ascorbate-free diet for 5 weeks to acute hypoxia (8% oxygen for 24h) was similar to Gulo$^{-/-}$ mice supplemeted with ascorbate, as measured by kidney Epo mRNA levels and plasma EPO protein levels. These data suggest that *in vivo* ascorbate is dispensible for functional oxygen sensing and possibly other antioxidants could compensate for the lack of ascorbate. Certainly further investigations are required to elucidate the molecular mechanism of glutathione (and other antioxidants) action on prolyl-4-hydroxylases activity. Of note, also other enzymes which depend on ascorbate supplementation for *in vitro* function like collagen prolyl-4-hydroxylases and enzymes involved in carnitine biosynthesis were reported to be independent of the ascorbate availability *in vivo* (Furusawa et al., 2008; Parsons et al., 2006).

11.3 Clinical application of antioxidants in the context of the HIF-PHD pathway

Besides their specific hydroxylation activity, also *de novo* expression and protein stability of PHDs are important factors that can influence overall oxygen sensing via the PHDs/HIF pathway. As it was shown previously, expression of PHD2 and PHD3 can be transcriptionally induced by HIFs, thus establishing a negative feedback loop which allows cells/tissues to adapt to chronic oxygen deficiency and still respond to a second insult of more severe hypoxia (Minamishima et al., 2009; Stiehl et al., 2006). Since PHDs and HIF are implicated in many pathological processes, modulation of their expression or activity would be of great interest in developing novel treatment regiments for these diseases. Many PHDs inhibitors could be used in treatment of disorders caused by insufficient EPO production. Administration of PHDs inhibitors to these patients could replace therapy using recombinant EPO injections as exogenous EPO often activates the immune system. Moreover, PHDs inhibitors can avoid parenteral EPO injections, as some of the new drugs might be applied orally.

In summary, we showed that oxygen sensing via PHDs is a tightly regulated process, which can be influenced by many extra- and intracellular factors. We could demonstrate that ascorbate is not essential for PHD activity *in vitro* and *in vivo* and can be replaced by glutathione. As ascorbate and N-acetyl-cysteine were proposed previously as antitumorigenic agents acting at least partially via the HIF pathway (Gao et al., 2007), glutathione might be similarly functional to modulate HIF activity in solid cancers. Moreover, combined treatment of different antioxidants could enhance their effects on PHDs and HIF. A drawback of antioxidants at pharmacological doses might be that most compounds - besides their considerably beneficial properties as radical scavengers - paradoxically also generate free radicals depending on concentration and the specific environment (Chen et al., 2005). Moreover, ascorbate and glutathione serve as co-factors for a number of enzymes in cellular systems, therefore their specificity towards PHDs is rather low. Nevertheless, the antioxidative properties of ascorbate or glutathione clearly affect PHD activity under certain conditions, and elucidating the underlying molecular events of PHD and ascorbate/glutathione interplay might open novel routes to specifically target the PHD/HIF oxygen sensing pathway.

References:

Chen Q, Espey MG, Krishna MC, Mitchell JB, Corpe CP, Buettner GR, Shacter E, Levine M (2005). Pharmacologic ascorbic acid concentrations selectively kill cancer cells: action as a pro-drug to deliver hydrogen peroxide to tissues. Proc Natl Acad Sci USA 102(38): 13604-9.

Epstein AC, Gleadle JM, McNeill LA, Hewitson KS, O'Rourke J, Mole DR, Mukherji M, Metzen E, Wilson MI, Dhanda A, Tian YM, Masson N, Hamilton DL, Jaakkola P, Barstead R, Hodgkin J, Maxwell PH, Pugh CW, Schofield CJ, Ratcliffe PJ (2001). C. elegans EGL-9 and mammalian homologs define a family of dioxygenases that regulate HIF by prolyl hydroxylation. Cell. 107(1): 43-54.

Flashman E, Davies SL, Yeoh KK, Schofield CJ (2010). Investigating the dependence of the hypoxia-inducible factor hydroxylases (factor inhibiting HIF and prolyl hydroxylase domain 2) on ascorbate and other reducing agents. Biochem 427(1): 135-42.

Furusawa H, Sato Y, Tanaka Y, Inai Y, Amano A, Iwama M, Kondo Y, Handa S, Murata A, Nishikimi M, Goto S, Maruyama N, Takahashi R, Ishigami A (2008). Vitamin C is not essential for carnitine biosynthesis in vivo: verification in vitamin C-depleted senescence marker protein-30/gluconolactonase knockout mice. Biol Pharm Bull. 31(9): 1673-9.

Gao P, Zhang H, Dinavahi R, Li F, Xiang Y, Raman V, Bhujwalla ZM, Felsher DW, Cheng L, Pevsner J, Lee LA, Semenza GL, Dang CV (2007). HIF-dependent antitumorigenic effect of antioxidants in vivo. Cancer Cell 12(3): 230-8.

Halliwell B, Gutteridge JM (2007). Free radicals in biology and medicine. Oxford University Press: p.184.

Hirsilä M, Koivunen P, Günzler V, Kivirikko KI, Myllyharju J (2003). Characterization of the human prolyl 4-hydroxylases that modify the hypoxia-inducible factor. J Biol Chem 278(33): 30772-80.

Hirsila M, Koivunen P, Xu L, Seeley T, Kivirikko KI, Myllyharju J (2005). Effect of desferrioxamine and metals on the hydroxylases in the oxygen sensing pathway. Faseb J. 19(10): 1308-10.

Kaelin WG, Jr., Ratcliffe PJ (2008). Oxygen sensing by metazoans: the central role of the HIF hydroxylase pathway. Mol Cell. 30(4): 393-402.

Maeda N, Hagihara H, Nakata Y, Hiller S, Wilder J, Reddick R (2000). Aortic wall damage in mice unable to synthesize ascorbic acid. Proc Natl Acad Sci USA 97(2): 841-6.

Mandl J, Szarka A, Banhegyi G (2009). Vitamin C: update on physiology and pharmacology. Br J Pharmacol. 157(7): 1097-110.

Martin F, Linden T, Katschinski DM, Oehme F, Flamme I, Mukhopadhyay CK, Eckhardt K, Troger J, Barth S, Camenisch G, Wenger RH (2005). Copper-dependent activation of hypoxia-inducible factor (HIF)-1: implications for ceruloplasmin regulation. Blood. 105(12): 4613-9.

Mecinovic J, Chowdhury R, Flashman E, Schofield CJ (2009). Use of mass spectrometry to probe the nucleophilicity of cysteinyl residues of prolyl hydroxylase domain 2. Anal Biochem 393(2): 215-21.

Minamishima YA, Moslehi J, Padera RF, Bronson RT, Liao R, Kaelin WG, Jr. (2009). A feedback loop involving the Phd3 prolyl hydroxylase tunes the mammalian hypoxic response in vivo. Mol Cell Biol. 29(21): 5729-41.

Nytko KJ, Spielmann P, Camenisch G, Wenger RH, Stiehl DP (2007). Regulated function of the prolyl-4-hydroxylase domain (PHD) oxygen sensor proteins. Antioxid Redox Signal 9(9): 1329-38.

Oehme F, Jonghaus W, Narouz-Ott L, Huetter J, Flamme I (2004). A nonradioactive 96-well plate assay for the detection of hypoxia-inducible factor prolyl hydroxylase activity. Anal Biochem. 330(1): 74-80.

Page EL, Chan DA, Giaccia AJ, Levine M, Richard DE (2008). Hypoxia-inducible factor-1alpha stabilization in nonhypoxic conditions: role of oxidation and intracellular ascorbate depletion. Mol Biol Cell. 19(1): 86-94. Epub 2007 Oct 17.

Pan Y, Mansfield KD, Bertozzi CC, Rudenko V, Chan DA, Giaccia AJ, Simon MC (2007). Multiple factors affecting cellular redox status and energy metabolism modulate hypoxia-inducible factor prolyl hydroxylase activity in vivo and in vitro. Mol Cell Biol 27(3): 912-25.

Parsons KK, Maeda N, Yamauchi M, Banes AJ, Koller BH (2006). Ascorbic acid-independent synthesis of collagen in mice. Am J Physiol Endocrinol Metab 290(6): E1131-9.

Salnikow K, Donald SP, Bruick RK, Zhitkovich A, Phang JM, Kasprzak KS (2004). Depletion of intracellular ascorbate by the carcinogenic metals nickel and cobalt results in the induction of hypoxic stress. J Biol Chem 279(39): 40337-44.

Stiehl DP, Wirthner R, Köditz J, Spielmann P, Camenisch G, Wenger RH (2006). Increased prolyl 4-hydroxylase domain proteins compensate for decreased oxygen levels. Evidence for an autoregulatory oxygen-sensing system. J Biol Chem 281(33): 23482-91.

12. Contributions to publications and manuscripts:

1. **Nytko KJ**, Spielmann P, Camenisch G, Wenger RH and Stiehl DP. Regulated Function of the Prolyl-4-hydroxylase Domain (PHD) Oxygen Sensor Proteins. Antioxid Redox Signal. 2007 Sep;9(9):1329-38. (Manuscript I).
   All figures except subfigure 4C.

2. Barth S, Nesper J, Hasgall PA, Wirthner R, **Nytko KJ**, Edlich F, Katschinski DM, Stiehl DP, Wenger RH and Camenish G. The peptidyl-prolyl cis/trans isomerase FKBP38 determines HIF prolyl-hydroxylase PHD2 protein stability. Mol Cell Biol, 2007 May;27(10):3758-68.
   Expression and purification of recombinant pVHL-ElonginB-ElonginC complex used for hydroxylation assay in Figure 4 C and D.

3. Chen N, Rinner O, Czernik D, **Nytko KJ**, Stiehl DP, Zamboni N, Gstaiger M and Frei C. The oxygen sensor PHD3 limits glycolysis under hypoxia via direct binding to Pyruvate kinase. Letter, Cell Res. 2011 Apr 12. [Epub ahead of print]. PMID: 21483450.
   Expression and purification of recombinant PHD3 and hydroxylation assay in Figure 1 .

4. **Nytko KJ**, Maeda N, Schläfli P, Spielmann P, Wenger RH and Stiehl DP. Vitamin C is dispensable for oxygen sensing *in vivo*. Blood, 2011 Feb 23. [Epub ahead of print]. PMID: 21346252.
   All Figures.

5. Lehmann S, Stiehl DP, Honer M, Dominietto M, Baltes C, Keist R, **Nytko KJ**, Ametamey S, Wenger RH and Rudin M. Chronic pharmacological inhibition of prolyl-4-hydroxylases downregulates hypoxia-inducible factor 1 activity in mouse tumor allografts. *In preparation*.
   Erythropoietin ELISA in Figure 1C and qPCR in Figure 5D.

13. *Curriculum Vitae*

Name	**Katarzyna Jozefa NYTKO**
Work Address	INSTITUTE OF PHYSIOLOGY UNIVERSITY OF ZÜRICH - IRCHEL WINTERTHURERSTRASSE 190 CH-8057 ZÜRICH SWITZERLAND
Private address	ALTWIESENSTRASSE 124 CH-8051 ZÜRICH SWITZERLAND
Telephone	044 635 5051 (work), 0763023328 (private)
E-mail	katarzyna.nytko@access.uzh.ch
Nationality	Polish
Date of birth	April 1, 1982

EDUCATION AND TRAINING

Since Sept. 2006	PhD Thesis University of Zürich, Institute of Physiology, Zürich, Switzerland PhD project title: "Regulated Function of Oxygen Sensing HIF Prolyl-4-Hydroxylases" Supervisors: Daniel P. Stiehl, Roland H. Wenger, Institute of Physiology, University of Zürich
Sept. 2005 – Febr. 2006	Socrates Erasmus Scholarship Laboratory for Stem Cell Research, Aalborg University, Denmark
Oct. 2001 – June 2006	Master studies, Master in Biology (majoring in Biochemistry) Jagiellonian Univeristy, Krakow, Poland Diploma Thesis title "The effect of hypoxia on differentiation of human and mouse mesenchymal stem cells", Supervisor: Hanna Rokita, Faculty of Biotechnology, Jagiellonian University
Sept. 1997 – June 2001	High School Certificate with Honors Kazimierz Brodzinski High School No. 1 in Tarnow, Poland

PERSONAL SKILLS AND COMPETENCES

LANGUAGES

Polish (native), English (fluent), German (proficient), Greek (basic)

TEACHING

Assistance in practical courses in physiology for medical and biology students (2006-2009)

CONFERENCES AND PRESENTATIONS

- Hypoxianet COST meeting – **poster presentation**, Davos, Switzerland, January 2011
- Keystone symposia: Molecular Mechanisms of Oxygen Sensing and Response Pathways – **poster presentation**, Keystone, Colorado, USA, January 2010
- Annual Meeting of the Swiss Physiological Society – **oral presentation** , Bern, Switzerland, September 2009
- 5th Symposium of the Zürich Center for Integrative Human Physiology (ZIHP) – **poster presentation**, Zürich, August 2009
- 43. Atmungsphysiologischen Arbeitstagung – **oral presentation,** University of Zürich, Zürich, January 2009
- 4th Symposium of the Zürich Center for Integrative Human Physiology (ZIHP) – **poster presentation**, Zürich, August 2008
- 3rd Retreat of the PhD programme in Integrative Molecular Medicine (imMed) – **oral presentation,** Magglingen, June 2008
- 87th Annual Meeting of the German Physiological Society (DPG) – **oral presentation,** Cologne, Germany, March 2008
- 3rd Symposium of the Zürich Center for Integrative Human Physiology (ZIHP) – **oral presentation**, Zürich, August 2007
- 6. Framework Programme of the EU/EUROXY course – **poster presentation**, Maastricht, Netherlands, February 2007
- "Oxygen Biology, Biotechnology and New EU rules", Oslo, Norway, January 2006
- "Innovative entrepreneurship, or how to turn innovation into business", Jagiellonian University, Krakow, Poland, 2005

PUBLICATIONS

1. Barth S., Nesper J., Hasgall P.A., Wirthner R., **Nytko K.J.**, Edlich F., Katschinski DM., Stiehl D.P., Wenger RH and Camenish G. The peptidyl-prolyl cis/trans isomerase FKBP38 determines HIF prolyl-hydroxylase PHD2 protein stability. Mol Cell Biol, 2007 May;27(10):3758-68.
2. **Nytko K.J.**, Spielmann P., Camenish G., Wenger R.H. and Stiehl D.P. Regulated function of the Prolyl 4-Hydroxylase Domain (PHD) Oxygen Sensor Proteins. Antioxid Redox Signal, 2007 Sep;9(9):1329-38.
3. Chen N, Rinner O., Czernik D, **Nytko K.J.**, Zheng D, Stiehl D.P., Zamboni N., Gstaiger M and Frei C. The oxygen sensor PHD3 limits glycolysis under hypoxia viadirect binding to Pyruvate kinase. Letter, Cell Res, 2011. PMID: 21483450
4. **Nytko K.J.**, Maeda N, Schläfli P, Spielmann P, Wenger RH and Stiehl DP. Vitamin C is dispensable for oxygen sensing *in vivo*. Blood, in press. PMID: 21346252.

14. Acknowledgments

I would like to express my gratitude to Prof. Dr. Roland Wenger for giving me the opportunity to perform a PhD Thesis in his laboratory as well as for his guidance and scientific support.

I would like to thank my supervisor Dr. med. Daniel Stiehl for his precious comments and ideas during my PhD Thesis as well as for the correcting and writing of the manuscripts.

I would like to thank all the members of my Thesis Committee: Dr. Felix Oehme, Prof. Dr. Christian Grimm and Dr. Gieri Camenisch for attending my Committee Meetings and sharing their comments and opinions. Special thanks to Dr. Felix Oehme for reviewing the Thesis as external expert.

I would like to thank Prof. Nobyo Maeda for providing mice and other materials, which were crucial for the outcome of the project.

Special thanks to Philipp Schläfli and Patrick Spielmann for help with the animal experiment. I would like to also thank Patrick for his technical assistance in the lab.

I would like to thank Beat Blattmann for a great help with HPLC measurements and Vinko Tosevski for technical assistance with FACS analyses.

I would like to thank my friends Magda, Peter, Adrian, Kamila, Darek, Hanna and João for all great moments outside the lab in Switzerland.

I would like to say thank you to my parents, my sister Magda and my brother Darek for always being there for me.

Last but not least I would like to thank Emmanuel for his constant support and patience.